Exhibit C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 98,385-A)

PATENT

In application of: Hauptmann *et al.*	)	**Before the Examiner: G. Draper**
	)	
Serial No.: 08484,312	)	**Group Art Unit: 1646**
	)	
Filed: June 7, 1995	)	
	)	
For: TNF RECEPTORS, TNF BINDING PROTEINS AND DNAs CODING FOR THEM	)	

DECLARATION PURSUANT TO 37 C.F.R SECTION 1.132

I, John D. Mountz, M.D. Ph.D., residing at 2800 Vestavia Forest Place, Birmingham, Alabama, United States of America, hereby declare:

1. I am Professor of Medicine in the Department of Medicine, Division of Clinical Immunology and Rheumatology, The University of Alabama at Birmingham. My Curriculum Vitae is attached hereto as **Appendix A.**

2. I have read U.S. Patent No. 5,695,953 (hereinafter, "the '953 patent"; attached hereto as **Appendix B**) and have considered the written description of the material disclosed in the patent and the extent to which this disclosure would enable one of ordinary skill to make and use recombinant DNA encoding a soluble tumor necrosis factor (TNF) inhibitor protein (hereinafter "TNF-IP").

Extent of Actual Experimental Disclosure in '953 Patent

3. The '953 patent discloses the existence of a TNF "inhibiting" activity in human urine (col. 4, ln. 9-10). This activity was associated with a protein having a molecular weight of 40-80 kilodaltons (kD) as determined by gel filtration chromatography (col. 4, ln. 10-13), termed TNF-IP.

4. The '953 patent describes generally a biochemical separation protocol for obtaining TNF-IP from urine of healthy individuals (col. 5, ln. 14-15). The biochemical separation protocol included membrane filtration to concentrate the urinary fluid (col. 8, ln. 30-37); two different cation-exchange chromatography steps (col. 8, ln. 40-59 and col. 8, ln. 61

through col. 9, ln. 15), anion-exchange chromatography (col. 9, ln. 16-35), and reversed-phase high pressure liquid chromatography (col. 9, ln. 36-64).

5. The protein product of this biochemical separation protocol was analyzed by SDS-polyacrylamide gel electrophoresis (col. 9, ln. 65 through col. 10, ln. 19) and yielded a band migrating at a molecular weight of about 26-28 kD (col. 10, ln. 17-19), and further described as being 27 kD in size (col. 10, ln. 37).

6. This putative inhibitor protein was able to inhibit the biological activity of TNFα as demonstrated by treating TNF-sensitive cells with TNFα in the presence of the inhibitor protein (col. 6, ln. 24-40 (Table I); col. 6, ln. 52 through col. 6, ln. 57).

7. This protein product was reported to be over 40% "pure" and was also described as being the "major" protein in the preparation (col. 10, ln. 35-37).

8. From this partially-purified protein preparation, an amino acid sequence was obtained that was represented as being fourteen of the first sixteen N-terminal amino acids of TNF-IP, with one residue being identified as theoretical and a second residue being unidentified (col. 10, ln. 21-56).

9. This is the extent of the actual disclosure of the '953 patent, *i.e.*, what was actually done by the inventors.

Prophetic Disclosure in '953 Patent

10. In addition to the actual disclosure, the '953 patent contained the prophetic description of additional experiments directed toward isolation of a recombinant DNA molecule encoding the TNF-IP product of the biochemical separation protocol (col. 10, ln. 57 through col. 16, ln. 10).

11. This disclosure concerned experiments that were proposed but never performed by the named inventors of the '953 patent.

12. The '953 patent speculates that cloning can be achieved by conventional techniques, including describing in the most general terms three proposed methods for isolating TNF-IP encoding cDNA: using antibodies to screen λgt11 cDNA libraries, using oligonucleotide probes to screen cDNA libraries or using oligonucleotide probes to screen genomic libraries.

13. The first two of these methods were absolutely dependent on identifying a cultured cell line expressing TNF-IP and preparing a cDNA library from such a cultured cell line

(col. 11, ln. 40 through col. 12, ln. 5). The '953 patent predicted that a cell line expressing TNF-IP could be found by either immunofluorescence detection or "Western" blotting of candidate cell lines using an antibody prepared from TNF-IP (col. 11, ln. 29).

14. The '953 patent recommended that such an antibody be prepared by injecting rabbits or mice with either the protein product of the biochemical separation protocol described in the patent, or by using a synthetic peptide corresponding to the N-terminal amino acid sequence (as reported at col. 10, ln. 43) (col. 11, ln. 17-24). In a suggested alternative, the '953 patent teaches that the putative TNF-IP N-terminal peptide fragment could be used to produce a fusion protein in *E. coli* that could be purified and injected into mice (col. 11, ln. 19-22).

15. Once a cell line expressing TNF-IP had been identified using this scheme, the '953 patent taught that a cDNA library would be prepared from cellular mRNA using conventional techniques (col. 11, ln. 39).

16. In the first method suggested by the '953 patent, TNF-IP cDNA would be cloned into a λgt11 cloning vector, which enables expression selection of desired clones (col. 11, ln. 42). The supposed advantage of using this vector was that an antibody, such as antibodies produced according to the teachings of the '953 specification, could be used to screen the cDNA library.

17. In the second method, the '953 patent taught that a cDNA library could be screened with probes produced from the predicted nucleotide sequence of the putative N-terminal peptide of the putative disclosed TNF-IP protein (col. 12, ln. 5-15).

18. The '953 patent also taught that additional peptide sequences could be obtained from the putative TNF-IP by proteolytic fragmentation using known proteases (col. 12, ln. 19).

19. The '953 patent taught that it may be possible to employ an unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the TNF-IP. The use of such oligonucleotide or set of oligonucleotides containing the theoretical "most probable" sequence capable of encoding the TNF Inhibitory Protein gene fragments (following the "codon usage rules" disclosed by Lathe *et al.*, 1985, *J. Molec. Biol.* 183: 1) permits one to identify the sequence of a complementary oligonucleotide or set of oligonucleotides which may be used as a probe for the gene of TNF-IP (col. 12, ln. 47 to col. 13, ln. 2).

20. The last of the three suggested methods disclosed in the '953 patent for isolating a DNA molecule encoding TNF-IP involved screening a library prepared from human genomic DNA with the same oligonucleotide probes as taught for screening cDNA libraries (col. 11, ln. 63 *et seq.*).

21. There was no disclosure of the results of any of the cloning experiments described in the '953 patent, and no evidence that any of these experiments had actually been performed at the time the application that matured into the '953 patent was filed.

22. Neither the nucleotide sequence nor production of any oligonucleotide, degenerate oligonucleotide or mixture of degenerate oligonucleotides was disclosed in the '953 patent.

23. The identity of no cell line expressing TNF-IP was disclosed in the '953 patent.

24. The screening of no cDNA or genomic DNA library was disclosed in the '953 patent, and no DNA molecule encoding TNF-IP was disclosed in the '953 patent.

Failure of Inventors to Successfully Clone DNA by Prophetic Teachings

25. In a series of patent publications and other publicly-available references, some or all of the inventors named in the '953 patent reported that several of their attempts to isolate a DNA molecule encoding TNF-IP according to the methods described in the '953 patent were unsuccessful.

26. For example, Israeli Patent Application No. IL 92697 (**Appendix C**) discloses the applicant's failure to obtain a cDNA encoding TNF-IP by screening oligo dT-primed cDNA libraries prepared from human liver and placental tissue, or the human cell lines HeLa and U937 using a mixture of degenerate oligonucleotides prepared as described in the '953 patent and derived from the putative N-terminal amino acid sequence of the putative TNF-IP protein described in the '953 patent.

27. In this application, presumably at least 150 positively-hybridizing clones were obtained in an initial screening. Of these clones, three survived additional rounds of screening by positively-hybridizing to the degenerate probe mixture. All three of these clones were found to be false-positives that did not encode TNF-IP when the DNA sequence of the clones was determined.

28. This negative result was obtained despite the fact that one of the methods disclosed in the '953 patent was followed in performing the cloning and screening experiments.

29. Moreover, this negative result was obtained even though it was later demonstrated by others that U937 cells produce an mRNA encoding TNF-IP as part of a larger transcript (EP 422339; **Appendix D**).

30. These researchers also screened a randomly-primed colon cDNA library using a degenerate probe mixture containing the nucleotide base analog inosine (which was not described in the '953 patent) and obtained a positively-hybridizing longer cDNA fragment.

31. This longer cDNA sequence is designated C2 in the reference and is present in the Israeli Patent Application (**Appendix C**). The C2 sequence was incomplete and devoid of a translational start codon or a stop codon. The C2 sequence also includes a number of bases that are different from the claimed sequences (see sequence comparison, **Appendix M**). Thus, the C2 sequence appears to be a related but distinct sequence from the claimed sequence.

32. The C2 sequence was used as a second probe for screening the colon cDNA library. The '953 patent did not disclose production of a secondary probe comprising what appears to be a distinct sequence for screening a cDNA library.

33. Even when using the longer C2 sequence as a probe, the attempt to identify a full-length clone encoding TNF-IP failed in two experiments: the first being a rescreening of the colon library from which the C2 sequence had been obtained, and the second being the screening of an oligo dT-primed placental cDNA library.

34. It was not until the experiments set forth in the publication of Nophar *et al.* (**Appendix E**) were performed that these inventors were able to produce a TNF-IP encoding cDNA clone. However, this success was achieved well after the effective filing date of the '953 patent.

35. The experiments described in the Nophar *et al.* reference required screening a λZAP cDNA library constructed from randomly-primed cDNA produced from human CEM cells.

36. The CEM library used by Nophar *et al.* was not available at the effective filing date of the '953 invention (*see* the Clontech catalogue dated 1998; **Appendix F**). This library only became available after the effective filing date of the '953 patent (*see* the Clontech catalog dated 1989-1990; **Appendix G**).

37. Nophar *et al.* does not teach why the CEM cDNA library was chosen, but CEM

cells apparently were not identified as being TNF-IP expressors using immunofluorescence or Western blotting, as described in the '953 patent.

38. The evidence from the Nophar *et al.* reference demonstrates that the specification of the '953 patent did not enable one having ordinary skill in the art to produce a recombinant DNA encoding TNF-IP.

39. Even the inventors of the '953 patent themselves were not able to isolate a TNF-IP encoding clone using the teachings of the '953 patent.

Attempts of Other Research Groups to Clone DNA

40. In addition, other well-funded, motivated researchers at companies such as Boehringer Ingelheim, Hoffman-La Roche, Genentech and Synergen attempted to clone the DNA. Although there were numerous failures by these groups to isolate a DNA molecule encoding TNF-IP using information at least as detailed as in the '953 patent, not one of those groups successfully isolated a DNA molecule encoding TNF-IP by simply using the information on the face of '953 patent.

41. As set forth below, each of these research groups was required to creatively resolve critical issues left unresolved by the teachings of the '953 patent. For example, each of the research groups (a) used affinity chromatography, a biochemical purification protocol different from that reported in the '953 patent; (b) generated and used additional amino acid sequence information to generate hybridization probes; and (c) identified and screened cell sources not identified in nor identifiable from the '953 patent.

42. For example, researchers at Hoffman-LaRoche reported their cloning of cDNA encoding the TNF receptor (Loetscher *et al.*, 1990, *Cell* 61: 351-359; **Appendix H**).

43. These researchers reported that "conventional cloning" using "relatively short, fully degenerate or longer best-guess oligonucleotides" for screening oligo dT-primed cDNA libraries was "technically difficult" and had not resulted in cloning cDNA encoding the TNF receptor or TNF-IP (p.356). Sources of mRNA for preparing cDNA libraries screened in these failed experiments included human placental tissue and the human cell line HL60 (p. 357).

44. These researchers only successfully obtained a cDNA clone encoding the TNF receptor after producing a 78bp cDNA fragment using the polymerase chain reaction (PCR) with

an N-terminal sequence and an internal peptide fragment from an affinity-purified preparation, and screening a human placenta λgt11 cDNA library with this probe.

45. The '953 patent does not disclose the sequence of any internal peptide fragment, and thus not the specific fragment used by the Hoffman-LaRoche researchers, for preparing oligonucleotide probes. Moreover, the '953 patent makes no mention of using PCR to produce either cDNA or probes for screening cDNA.

46 The researchers did not state that the placental tissue was identified as being TNF-IP expressors using immunofluorescence or Western blotting, as described in the '953 patent.

47. The '953 patent does not identify placental tissue as providing a cell source of TNF-IP expressors.

48. Thus, the evidence from the Loetscher *et al.* reference is that these researchers were successful in obtaining a cDNA clone encoding TNF-IP only when (a) they obtained a 78bp, PCR-produced cDNA fragment, which was not disclosed in the patent and (b) they used the fragment to screen a human placenta λgt11 cDNA library, which was not disclosed in the '953 patent.

49. In another example, researchers at Boehringer Ingelheim screened oligo dT-primed cDNA libraries prepared from placenta and human cell lines U937, Hs913T and HeLa using mixtures of degenerate oligonucleotide probes derived from the N-terminal amino acid sequence from an affinity-purified preparation disclosed in the '953 patent (European Patent Application EP417563; **Appendix I**).

50. These experiments failed to produce a cDNA encoding TNF-IP.

51. It was only when these researchers screened an Hs913T library with a PCR fragment using the amino acid sequence of an internal tryptic peptide fragment from an affinity-purified preparation that a cDNA encoding TNF-IP was obtained.

52. The '953 patent does not disclose the sequence of any internal peptide fragment, and thus not the specific fragment used by the Boehringer Ingelheim researchers, for preparing oligonucleotide probes. Moreover, the '953 patent does not disclose cDNA cloning or screening methods using the PCR.

53. The researchers did not state that the Hs913T cells were identified as being TNF-IP expressors using immunofluorescence or Western blotting, as described in the '953 patent.

54. The '953 patent does not identify Hs913T cells as being cells that express TNF-IP.

55. Thus, the evidence from European Patent Application EP417563 is that these researchers were successful in obtaining a cDNA clone encoding TNF-IP only when (a) they obtained a PCR-produced fragment corresponding to an internal, protease-generated peptide, which was not disclosed in the '953 patent and (b) they used the fragment to screen an oligo dT-primed Hs913T cDNA library, which was not disclosed in the '953 patent.

56. In yet another example, researchers at Genentech reported their cloning of cDNA encoding the TNF receptor (Schall *et al.*, 1990, *Cell* 61: 361-370; **Appendix J**).

57. These researchers produced two purified, internal proteolytic fragments from an affinity-purified preparation that were used to prepare oligonucleotides for screening placental and HL60 cDNA libraries made in λgt10 (p. 368)

58. The Genentech researchers obtained four positively-hybridizing clones from a randomly-primed library and a single positively-hybridizing clone from an oligo dT-primed library.

59. The oligonucleotide probes used to screen these libraries were not degenerate, being specifically derived from less polymorphic regions of the protein and using the codon usage rules of Lathe *et al.* (p. 368).

60. The '953 patent does not identify the sequence of any internal peptide fragment for preparing non-degenerate oligonucleotide probes, and thus not the specific fragment used by the Genentech researchers, for preparing oligonucleotide probes

61. The researchers did not state that the HL60 cells were identified as being TNF-IP expressors using immunofluorescence or Western blotting, as described in the '953 patent.

62. The '953 patent does not identify HL60 cells as being cells that express TNF-IP.

63. Thus, the evidence from the Schall *et al.* reference is that these researchers were successful in obtaining a cDNA clone encoding TNF-IP only when they (a) used a cDNA library produced from a tissue (placenta) and a cell line (HL60) that were not first identified as TNF-IP expressors using antibodies according to the teachings of the '953 patent and (b) a sequence encoding an internal peptide that was not identified in the patent.

64. In a still further example, researchers at Synergen reported the cloning of cDNA

encoding the TNF receptor (EP 422339; **Appendix D**).

65. These researchers produced purified proteolytic fragments from an affinity-purified preparation that was used to prepare four degenerate oligonucleotide probes encoding N-terminal and internal peptide sequences for screening a genomic library (p. 23, ln. 32-52).

66. Initially an N-terminal probe identified eleven positive clones, with one clone hybridizing with all four degenerate probes (p. 23, ln. 55 through p. 24, ln. 14). The one clone was sequenced and encompassed significantly more of the N-terminal sequence (68 amino acids) from that which was taught in the '953 patent (p. 24, ln. 15-29).

67. The Synergen researchers used a probe encoding an internal peptide sequence of TNF-BP to screen an oligo dT-primed cDNA of U937 cells, from which three clones were identified (p. 24, ln. 49-53). The three plaques were confirmed with a probe encoding a second internal peptide sequence (p. 24, ln. 52-53).

68. The '953 patent does not disclose the sequence of any internal peptide fragment, and thus not the specific fragment used by the Synergen researchers, for preparing oligonucleotide probes.

69. The researchers did not identify U937 cells as being TNF-IP expressors using immunofluorescence or Western blotting, as described in the '953 patent.

70. The '953 patent does not identify U937 cells as being cells that express TNF-IP. In fact, a later-published reference by some of the inventors of the '953 patent support Applicants' position that the teachings of '953 may not have identified U937 cells as a suitable cell source.

71. The reference teaches anti-TNF-IP antibodies which were produced after immunization of mice with a TNF-IP preparation purified using a substantially different purification protocol that included affinity chromatography with immobilized TNFα (Engelmann *et al.*, 1989, *J. Biol. Chem.* 264: 11970-11980; **Appendix K**). The monoclonal antibodies produced according to the Engelmann *et al.* reference failed to bind to U937 cells.

72. Thus, the evidence from the EP 422339 is that these researchers were successful in obtaining a cDNA clone encoding TNF-IP only when they used (a) a cDNA library produced from a cell line (HL60) that was not first identified as TNF-IP expressors using antibodies according to the teachings of the '953 patent and (b) sequence encoding an internal peptide that

was not identified in the patent.

Conclusion

73. In my opinion, the disclosure of the '953 patent would not have enabled one of ordinary skill in the art to obtain a DNA molecule encoding TNF-IP for at least the following reasons.

74. The '953 patent does not enable one of ordinary skill in the art to obtain a cDNA molecule encoding TNF-IP because, at the effective filing date of the patent, no tissue or cell line was known that expressed TNF-IP.

75. The fact that TNF-IP is a proteolytic cleavage product of the TNF receptor, and represents the extracellular domain of the receptor, was not known at the effective filing date of the '953 patent (*see, for example*, Wallach *et al.*, 1989, *Lymphokine Research* 8: 361, which says:

"The cellular source of this urine-derived TNF binding protein remains to be elucidated." (**Appendix L**)

76. The protein prepared from the biochemical separation protocol described in the '953 patent is very impure, being >50% contaminated with other proteins.

77. This level of impurity would make the protein preparation described in the '953 patent difficult to use in preparing and screening antibodies specific for TNF-IP.

78. Also, this level of impurity would have led one of ordinary skill to believe that the peptide sequence disclosed in the '953 patent could not be relied upon as being related to the TNF-IP, since there was a better than even chance that this sequence was completely unrelated to the TNF inhibiting activity.

79. In addition, until a cDNA clone is isolated and a recombinant TNF-IP protein produced and characterized, it could not be established that the protein from which the N-terminal amino acid sequence was obtained in fact binds to TNF.

80. No cDNA libraries or sequence of probes encoding the putative N-terminal amino acid sequence were disclosed in the '953 patent.

81. The '953 patent only provides a limited amount of sequence information and an encyclopedia of experimental options. After having spent considerable time and effort in discovering that essential information was lacking in the patent, I would have been left with myriad potential research avenues but no specific guideposts of how to successfully navigate

them. In fact, each research group that successfully cloned the DNA relied on insights and methods that were not described in the '953 patent.

82. In summary, it is clear that the amino acid sequence disclosed in the patent and the absence of an identified cell source in the patent provided insufficient information to clone the DNA.

83. Thus, it is my opinion that one of ordinary skill in the art would not have been able to obtain a DNA molecule encoding TNF-IP using the methods set forth in the '953 patent.

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: _____
John D. Mountz, M.D. Ph.D.

Dated: 2/25/99

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Attorney Docket No. 98,385-A)

In the Application of:	)	
	)	
Hauptmann et al.	)	**Examiner: G. Draper**
	)	
Serial No: 08/484,312	)	**Group Art Unit: 1646**
	)	
Filed: June 7, 1995	)	
	)	
For: TNF Receptors, TNF Binding Proteins and DNAs Coding for them	)	

Asst. Commissioner for Patent
Washington, DC 20231

TRANSMITTAL LETTER

In regard to the above-identified patent application:

1. We are transmitting herewith the attached:
   a. Amendment and Response
   b. Attached Exhibits
   c. Postcard.

2. With respect to additional fees:

   _X_ A. No additional fee is required.

   _____ B. Attached are two checks in the amount of _____

3. Please charge any additional fees or credit over-payments to the Deposit Account No.13-2490.

4. _x_ CERTIFICATE UNDER 37 CFR 1.18: The undersigned hereby certifies that this Transmittal Letter and this paper, as described in paragraph 1 hereinabove, are being hand delivered to: Examiner G. Draper in Group Art Unit 1646, Washington, D.C. 20231, on this 1st day of March 1999.

Dated: March 1, 1999 By: _____

John J. McDonnell
Reg. 26,949

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Appendix A

Updated 08/20/98

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
SCHOOL OF MEDICINE
FACULTY CURRICULUM VITAE

PERSONAL INFORMATION

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HOSPITAL APPPOINTMENTS:

University of Alabama Hospitals
Veterans Administration Hospital

PROFESSIONAL CONSULTANTSHIPS:

Updated 08/20/98

Consultant Scientist; Marion Merrell Dow, Inc.; Kansas City, MO
Consultant Scientist; Wyeth-Ayerst, Princeton, NJ
Consultant Scientist; LXR Biotechnologies, Inc., Richmond, CA
Consultant Scientist; Amgen Boulder, Inc., Boulder, CO

EDUCATION:

Institution	Degree	Year
Wright State University, Dayton, OH	BS	1971
Michigan State University, East Lansing, MI	MS	1971
Michigan State University, East Lansing, MI	PhD	1974
Ohio State University, Columbus, OH	MD	1978

MILITARY SERVICE: N/A

LICENSURE:

Physician, North Carolina #23624
Physician, Alabama #13416

BOARD CERTIFICATION:

American Board of Internal Medicine - 1982

POSTDOCTORAL TRAINING:

Year	Degree	Institution
1974-1975	Postdoctoral Fellow	National Science Foundation East Lansing, MI
	Medical School Scholarships	William Craig-Orr and Mary Black-Orr Scholarship in Medicine
		Kenneth Wiseman Scholarship in Medicine
1978-1981	Intern and Resident	Internal Medicine Residency Program; North Carolina Baptist Hospital
1981-1982	Rheumatology Fellow	Bowman Gray School of Medicine
1982-1987	Medical Staff Fellowship	National Institutes of Health; National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases
1988-present	Director	Transgenic Mouse Facility, Birmingham Veterans Administration Medical Center

ACADEMIC APPOINTMENTS:

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Year	Rank/Title	Institution
1985-1987	Research Assistant Professor of Medicine	Uniformed Services University of Health Sciences
1986-1987	Guest Researcher	National Institutes of Health; National Institute of Arthritis and Musculoskeletal and Skin Diseases
1987-1991	Assistant Professor of Medicine Division of Clinical Immunology and Rheumatology	UAB
1991-1994	Associate Professor of Medicine Division of Clinical Immunology and Rheumatology	UAB
1991-present	Associate Professor, Division of Gerontology and Geriatric Medicine	UAB
1991-present	Associate Professor, Cellular & Molecular Biology, Department of Microbiology (Joint appointment renewed for 3 years March, 1996)	UAB
1987-present	Staff Physician	Birmingham VAMC
1993-present	Scientist, Medical Center Joint Departments, Center for Aging	UAB
1994-present	Professor of Medicine, Division of Clinical Immunology and Rheumatology	UAB

HONORS AND AWARDS:

B.S., Summa Cum Laude
M.S., Summa Cum Laude
Phi Eta Tau - Scholastic Honor Society
Elected to Who's Who of Birmingham - 1990

AWARDS FOR RESEARCH ACCOMPLISHMENTS:

Senior Rheumatology Scholar Award - 1986
Howard and Martha Holley Research Prize in Rheumatology - March 13, 1992

MEMBERSHIPS AND OFFICES IN PROFESSIONAL SOCIETIES:

American College of Physicians - 1978
American Association of Immunology - 1985
American College of Rheumatology - 1986
Southern Society of Clinical Investigation - 1988
American Federation for Clinical Research - 1990

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American Society of Internal Medicine - 1990
American Society of Clinical Investigation - 1992
Southern Medical Association - 1992
Association of American Physicians - 1997

COUNCILS AND COMMITTEES:

COMMITTEES

Arthritis and Prostaglandins Research Challenge
Advisory Meeting. Searle Pharmaceuticals, Chicago, IL. May 3-6, 1990.
Veterans Administration Transgenic Mouse Facility Scientific Advisory Committee (1990-)
Veterans Administration Medical Center Transgenic Mouse Subcommittee (1990-)
Veterans Administration Animal Use Committee (1991-)
Department of Medicine Research Committee (1991-)
Admissions Committee, School of Medicine, University of Alabama at Birmingham, Department of Medicine, MD, PhD. Training Program (1991-)
Research Council of American College of Physicians (1993-1996)
Geriatrics Research Center Advisory Committee (1993-)
Chairman, Adhesion Molecule and Cell Matrix Center Director Search Committee (1993)
Committee for Revision of American College of Rheumatology Patient Care Summary (1993-1994)
Research Committee of the ACR Research and Education Foundation (1994-)
Co-Chairs, Task Force, Committee, National Institute on Aging, National Institute of Allergy and Infectious Diseases Task Force on Immunology and Aging
Veterans Administration Merit and Career Development Review Committee; Immunology Study Section, (1995-1998)
Chairman, Department of Medicine Research Committee (1996-)
UAB/Sankyo Co., Ltd., Program for Rheumatic Diseases Oversight Committee (1996)

UNIVERSITY ACTIVITIES:

Department of Medicine Research Committee (1991-)
Veterans Administration Transgenic Mouse Facility Scientific Advisory Committee (1990-)
Veterans Administration Medical Center Transgenic Mouse Subcommittee (1990-)
Veterans Administration Animal Use Committee (1991-)
Admissions Committee, School of Medicine, University of Alabama at Birmingham, Department of Medicine, MD, PhD. Training Program (1991-)
Geriatrics Research Center Advisory Committee (1993-)
Chairman, Adhesion Molecule and Cell Matrix Center Director Search Committee (1993)
Chairman, Department of Medicine Research Committee (1996-)
UAB/Sankyo Co., Ltd., Program for Rheumatic Diseases Oversight Committee (1996)

Updated 08/20/98

EDITORIAL BOARDS/AD HOC REVIEWER:

Editorial Board, *Arthritis & Rheumatism*; 1988 - 1993
Editorial Board, *Cellular Immunology;* 1996 - 2000
Reviewer, *American Association of Immunologist*
Reviewer, *American Journal of Medicine*
Reviewer, *Autoimmunity*
Reviewer, *Blood*
Reviewer, *Antiviral Research*
Reviewer, *Clinical Chemistry*
Reviewer, *Gastroenterology*
Reviewer, *Human Immunology*
Reviewer, *International Immunology*
Reviewer, *Immunity*
Reviewer, *Immunology Today*
Reviewer, *Journal of Clinical Immunology*
Reviewer, *Journal of Clinical Investigation*
Reviewer, *Journal of Rheumatology*
Reviewer, *Science*
Reviewer, *Transgenes and Transgenic Mice*
Reviewer, *Transgenic Technology*
Reviewer, *FASEB Journal*

Reviewer, Veterans Administration Health Services and Research Administration, Career Development Program Applications, Study Section, National Arthritis Foundation (1991-1993)
Immunological Sciences Study Section Ad Hoc Reviewer, NIAID, June 9-11, 1993
External Reviewer, Medical Research Service, Department of Veterans Affairs. 1995-1997
Ad Hoc Reviewer, The Israel Science Foundation, Israel Academy of Science and Humanities, Jerusalem, Israel, March, 1996.
Grant Reviewer, The Arthritis and Rheumatism Council, 1997
Reviewer, The Wellcome Foundation, 1997
Immunological Sciences Study Section, 1997-2000

Site Visits:

Special Project Reviewer for NIH - U. California at San Diego Multipurpose and Musculoskeletal Disease Center (MAMDC) 1991
Special Project Reviewer for NIH, U. Texas at Dallas, MAMDC, 1991
Special Project Reviewer, Boston University School of Medicine, March 1996
Reviewer, Cornell Aging Program Project, December, 1996
Reviewer, University of Michigan Aging Program Project, February 1998.
Reviewer, " Cellular Senescence and Control of Proliferation" Allegheny University of the Health Sciences, Phildelphia, PA, July 28, 1998.

Updated 08/20/98

MAJOR LECTURES AND VISITING PROFESSORSHIPS:

1. "Therapies which reduce lymphadenopathy and autoimmunity of MRL/*lpr* Mice: Implications for the Pathogenesis and Treatment of RA and SLE," New York Academy of Sciences, November 12, 1987.
2. "The Role of T Cells in Autoimmune Disease," Lowe Southeastern Conference on Rheumatic Diseases, September 28, 1990.
3. "Update on Arthritis Research," Arthritis Foundation Dinner, 3rd Humanitarian Award Ceremony, September 21, 1990.
4. "A Superantigen Model for Arthritis in V_b8 TCR Transgenic *lpr/lpr* Mice," Keystone Symposia on the Molecular Biology and the Immunopathogenesis of Rheumatoid Arthritis, March 16, 1991.
5. "Loss of T-cell Anergy in Autoimmunity Mice," Bar Harbor Symposia on MHC and TCR in Autoimmune Disease, October 18-20, 1991, Bar Harbor, Maine.
6. "T Cell Tolerance Defects in Transgenic Mice," Holley Symposia at Regional ACR Meeting, March 13, 1992.
7. "ETn Mutation in *Fas* CD2-*fas* Correction of *lpr* Mice," Keystone Symposia on the Molecular Biology and the Immunopathogenesis of Rheumatoid Arthritis, March 16, 1993.
8. "TCR Transgenic Mice and CD2-*Fas* Transgenic Mice," Autoimmunity Symposium, Bergen, Norway, April 23, 1993.
9. "Cellular and Molecular Mechanisms of Self Tolerance Loss in Autoimmunity," presented Medical Grand Rounds, November 11, 1993 at The University of Texas Health Science Center, San Antonio, Texas.
10. "Correction of T Cells and Autoimmune Disease in CD2-*fas* Transgenic Mice," FASEB Symposium June 14, 1993, Saxton River, VT.
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12. "T Cell Receptor Transgenic Autoimmune Mice," Oxford Conference on Autoimmunity. Presented at Trinity College, Oxford, England, September 20, 1993.
13. "T Cell Tolerance Loss and Aging". Presented at the Gordon Conference on The Biology of Aging, Casa Sirena (Oxford), CA, February 27 - March 4, 1994.
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Updated 08/20/98

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21. "Defective Expression of Hematopoietic Cell Protein-Tyrosine-Phosphatase (HCP) Blocks Fas-Mediated Apoptosis". Keystone Symposium in Tammaron, Colorado, March 5-11, 1995.
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23. "Soluble Fas and Apoptosis Defects in SLE". Presented at the 4th International Conference on SLE in Jerusalem, Israel, March 26-31, 1995.
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26. "Lifespan studies of transgenic mice expressing the Fas apoptosis gene". Presented at the Gordon Research Conferences "Biology of Aging" Meeting in Lucca, Italy, May 7-12, 1995.
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28. "Role of Fas in T Cell Development, Tolerance and Autoimmunity". Presented at the 9th International Congress of Immunology Pathogenesis of Autoimmune Diseases Meeting in San Francisco, California, July 23-29, 1995.
29. "Aging and Apoptosis". Presented at the 48th Annual Scientific Meeting of The Gerontological Society of America Meeting, Los Angeles, California, November 15-19, 1995.
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44. Apoptosis and Autoimmunity. Presented at the American Academy of Allergy, Asthma and Immunology (AAAAI) , Washington, D.C., March 13-18, 1998.
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5. "Delta Nurr77 Tg Mice" , Mountz, Zhou, Cheng, Provisional Patent Filed May 13, 1996., Foreign Patent filed 5/06/97.
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Appendix B

US005695953A

United States Patent [19]

Wallach et al.

[11] Patent Number: 5,695,953

[45] Date of Patent: Dec. 9, 1997

[54] **DNA THAT ENCODES A TUMOR NECROSIS FACTOR INHIBITORY PROTEIN AND A RECOMBINANT METHOD OF PRODUCTION**

[75] Inventors: **David Wallach**, Rehovot, Israel; **Hartmut Engelmann**, Munich, Germany; **Dan Aderka**, Holon; **Menachem Rubinstein**, Givat Schmuel, both of Israel

[73] Assignee: **Yeda Research and Development Co. Ltd.**, Rehovot, Israel

[21] Appl. No.: **876,828**

[22] Filed: **Apr. 30, 1992**

Related U.S. Application Data

[63] Continuation of Ser. No. 243,092, Sep. 12, 1988, abandoned.

[30] **Foreign Application Priority Data**

Sep. 13, 1987 [IL] Israel 83878

[51] **Int. Cl.**[6] **C12P 21/06**; C07H 21/00

[52] **U.S. Cl.** **435/69.1**; 435/69.5; 435/252.3; 435/252.33; 435/320.1; 536/23.5

[58] **Field of Search** 435/69.5, 172.1, 435/320.1, 69.1; 530/351; 436/501; 935/12, 15; 536/23.5

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Attorney, Agent, or Firm—Browdy and Neimark

[57] **ABSTRACT**

Tumor Necrosis Factor (TNF) Inhibitory Protein is isolated and substantially purified and the DNA that encodes the TNF inhibitory protein, vectors, host cells, and a recombinant method for producing the encoded protein are also set forth. It has the ability to inhibit: (a) the binding of TNF to its receptors, and (b) the cytotoxic effect of TNF. TNF Inhibitory Protein and salts, functional derivatives and active fractions thereof can be used to antagonize the deleterious effects of TNF.

15 Claims, 5 Drawing Sheets

Appendix C

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hauptman, et al.

(ii) TITLE OF INVENTION: TNF RECEPTORS, TNF BINDING BINDING PROTEINS, AND DNAs CODING FOR THEM

(iii) NUMBER OF SEQUENCES:64

(iv) CORRESPONDENCE ADDRESS:
(A)ADDRESSEE:John J. McDonnell
(B)STREET:300 S. Wacker Drive
(C)CITY:Chicago
(D)STATE:IL
(E)COUNTRY:USA
(F)ZIP:60606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE:ASCII

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:08/484,312
(B) FILING DATE:June 7, 1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME:John J. McDonnell
(B) REGISTRATION NUMBER: 26,949
(C) REFERENCE/DOCKET NUMBER:98,385-A

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE:312-913-0001
(B) TELEFAX:312-913-9808

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:1365 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCCGTTGCGC TGGAAGGAAT TCGTGCGGCG CCTAGGGCTG AGCGACCACG 1150
AGATCGATCG GCTGGAGCTG CAGAACGGGC GCTGCCTGCG CGAGGCGCAA 1200
TACAGCATGC TGGCGACCTG GAGGCGGCGC ACGCCGCGGC GCGAGGCCAC 1250
GCTGGAGCTG CTGGGACGCG TGCTCCGCGA CATGGACCTG CTGGGCTGCC 1300
TGGAGGACAT CGAGGAGGCG CTTTGCGGCC CCGCCGCCCT CCCGCCCGCG 1350
CCCAGTCTTC TCAGATGA 1365

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:483 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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TTGCTGTACC AAGTGCCACA AAGGAACCTA CTTGTACAAT GACTGTCCAG 100
GCCCGGGGCA GGATACGGAC TGCAGGGAGT GTGAGAGCGG CTCCTTCACC 150
GCTTCAGAAA ACCACCTCAG ACACTGCCTC AGCTGCTCCA AATGCCGAAA 200
GGAAATGGGT CAGGTGGAGA TCTCTTCTTG CACAGTGGAC CGGGACACCG 250
TGTGTGGCTG CAGGAAGAAC CAGTACCGGC ATTATTGGAG TGAAAACCTT 300

TTCCAGTGCT TCAATTGCAG CCTCTGCCTC AATGGGACCG TGCACCTCTC 350
CTGCCAGGAG AAACAGAACA CCGTGTGCAC CTGCCATGCA GGTTTCTTTC 400
TAAGAGAAAA CGAGTGTGTC TCCTGTAGTA ACTGTAAGAA AAGCCTGGAG 450
TGCACGAAGT TGTGCCTACC CCAGATTGAG AAT 483

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:455 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu
5 10 15
Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu
20 25 30
Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
35 40 45
Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
50 55 60
Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
65 70 75
Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr
80 85 90
Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys
95 100 105
Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
110 115 120
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr
125 130 135
Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu
140 145 150
Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val
155 160 165
Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
170 175 180
Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys
185 190 195
Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
200 205 210
Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu
215 220 225
Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
230 235 240
Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys
245 250 255

```
Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn
                260                 265                 270
Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe
                275                 280                 285
Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr
                290                 295                 300
Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala
                305                 310                 315
Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala
                320                 325                 330
Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys Trp Glu Asp Ser Ala
                335                 340                 345
His Lys Pro Gln Ser Leu Asp Thr Asp Asp Pro Ala Thr Leu Tyr
                350                 355                 360
Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp Lys Glu Phe Val
                365                 370                 375
Arg Arg Leu Gly Leu Ser Asp His Glu Ile Asp Arg Leu Glu Leu
                380                 385                 390
Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln Tyr Ser Met Leu Ala
                395                 400                 405
Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala Thr Leu Glu Leu
                410                 415                 420
Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys Leu Glu
                425                 430                 435
Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro Pro Ala
                440                 445                 450
Pro Ser Leu Leu Arg
                455
```

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:161 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn
                 5                  10                  15
Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn
                20                  25                  30
Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu
                35                  40                  45
Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu
                50                  55                  60
Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser
                65                  70                  75
```

```
Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg Lys Asn
                 80                  85                  90
Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn
                 95                 100                 105
Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
                110                 115                 120
Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg
                125                 130                 135
Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu
                140                 145                 150
Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn
                155                 160
```

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:157 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CAGGGGAAA ATATTCACCC TCAAATAAT TCGATTTGCT GTACCAAGTG      50
CCACAAAGG AAACTACTTG TACAATGAC TGTCCAGGCC CGGGGCAGGA     100
TACGGACTG CAGGGAGTGT GAGAGCGGC TCCTTCACAG CCTCAGAAAA     150
CAACAAG                                                  157
```

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:13 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Asp Ser Val Xaa Pro Gln Gly Lys Tyr Ile His Pro Gln
                 5                  10
```

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:11 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys
5 10

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:12 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys
5 10

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:13 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Ile His Pro Gln Xaa Asn Ser Ile Xaa Xaa Xaa Lys
5 10

11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:14 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn Asn Lys
5 10

(12) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:15 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg
5 10 15

(13) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:13 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Thr Tyr Ley Tyr Asn Asp Cys Pro Gly Pro Gly Gln
5 10

(14) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:13 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Glu Met Gly Gln Val Glu Ile Ser Xaa Xaa Xaa Val Asp
5 10

(15) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg
5 10 15
Asp Thr Val Cys Gly
20

(16) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:19 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Tyr Ile His Pro Gln Xaa Asn Ser Ile Cys Cys Thr Lys Cys His
5 10 15
Lys Gly Xaa Tyr

(17) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:18 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr
5 10 15
Xaa Xaa Arg

(18) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:14 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gln Asn Thr Val Cys Thr Xaa His Ala Gly Phe Phe Leu Arg
5 10

(19) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:14 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn
5 10

(20) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:13 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln
5 10

(21) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAAGGTAAAT ATATTCATCC 20

(22) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAGGGTAAGT ACATCCATCC 20

(23) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CAAGGTAAAT ATATACATCC 20

(24) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAAGGCAAAT ATATTCATCC 20

(25) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CAGGGCAAGT ACATCCACCC 20

(26) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CAAGGCAAAT ATATACATCC 20

(27) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CAAGGAAAAT ATATTCATCC 20

(28) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CAGGGAAAGT ACATCCACCC 20

(29) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CAAGGAAAAT ATATACATCC 20

(30) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CAAGGGAAAT ATATTCATCC 20

(31) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CAGGGGAAGT ACATCCACCC 20

(31) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:20 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CAAGGGAAAT ATATACATCC 20

(33) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:14 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Glu Cys Gly Ser Gly Ser Phe Thr Ala Ser Glu Asn Asn Lys
5 10

(34) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:14 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Glu Cys Gly Ser Gly Ser Phe Thr Ala Ser Cys Asn Asn Lys
5 10

(35) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AAATGACGGA GACTCTTGTT GTTCCTAGGG 30

(36) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

AAGTGGCGTA GTCTTTTGTT GTTCCTAGGG 30

(37) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AAATGTCGGA GACTCTTGTT GTTCCTAGGG 30

(38) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

AAATGACGGT CACTCTTGTT GTTCCTAGGG 30

(39) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

AAGTGGCGTT CTCTTTTGTT GTTCCTAGGG 30

(40) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AAATGTCGGT CACTCTTGTT GTTCCTAGGG 30

(41) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AAATGACGGA GAACATTGTT GTTCCTAGGG 30

(42) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AAGTGGCGTA GTACTTTGTT GTTCCTAGGG 30

(43) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

AAATGTCGGA GAACATTGTT GTTCCTAGGG 30

(44) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

AAATGACGGT CAACATTGTT GTTCCTAGGG 30

(45) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

AAGTGGCGTT CTACTTTGTT GTTCCTAGGG 30

(46) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AAATGTCGGT CAACATTGTT GTTCCTAGGG 30

(47) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:51 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
5 10 15
Lys Cys His Lys Gly Thr Tyr Ley Tyr Asn Asp Cys Pro Gly Pro
20 25 30
Gly Gln Asp Thr Asp Cys Arg Gly Cys Glu Ser Gly Ser Phe Thr
35 40 45
Ala Ser Glu Asn Asn Lys
50

(48) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:158 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CAGGGGAAAT ATATTCACCC TCAAAATAAT TCGATTTCGT GTACCAAGTC 50
GCACAAAGGA ACCTACTTGT ACAATGACTG TCCAGGCCCG GGGCAGGATA 100
CGGACTGCAG GGAGTGTGAG AGCGGCTCCT TCACAGCCTC AGAAAACAAC 150
AAGGATCC 158

(49) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:26 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GGAATTCAGC CTGAATGGCG AATGGG 26

(50) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:25 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CCTCGAGCGT TGCTGGCGTT TTTCC 25

(51) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:23 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GGTCGACATT GATTATTGAC TAG 23

(52) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:23 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GGAATTCCCT AGGAATACAG CGG 23

(53) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:18 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GGCAAGGGCA GCAGCCGG 18

(54) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:53 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

AGCTTCTGCA GGTCGACATC GATGGATCGG TACCTCGAGC GGCCGCGAAT 50
TCT 53

(55) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:54 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CTAGAGAATT CGCGGCCGCT CGAGGTACCG GATCCATCGA TGTCGACCTG 50
CAGA 54

(56) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:63 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

AGCTCTAGAG ATTCGCGGCC GCTCGAGGTA CCGGATCCAT CGATGTCGAC 50
CTGCAGAAGC TTG 63

(57) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:64 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CTAGCAAGCT TCTGCAGGTC GACATCGATG GATCCGGTAC CTCGAGCGGC 50
CGCGAATTCT CTAG 64

(58) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:25 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

CAGGATCCGA GTCTCAACCC TCAAC 25

(59) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:43 bases

(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GGGAATTCCT TATCAATTCT CAATCTGGGG TAGGCACAAC TTC 43

(60) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:81 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

CACAGTCGAC TTACATTTGC TTCTGACACA ACTGTGTTCA CTAGCAACCT 50
CAAACAGACA CCATGGGCCT CTCCACCGTG C 81

(61) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:17 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GAGGCTGCAA TTGAAGC 17

(62) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:17 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

ATTCGTGCGG CGCCTAG 17

(63) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:17 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

GTCGGTAGCA CCAAGGA 17

(64) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:17 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

GTTTTCCCAG TCACGAC 17

(65) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:30 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GTCCAATTAT GTCACACC 18

(66) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:1334 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

GAATTCTCTG GACTGAGGCT CCAGTTCTGG CCTTTGGGGT TCAAGATCAC 50
TGGGACCAGG CCGTGATCTC TATGCCCGAG TCTCAACCCT CAACTGTCAC 100

```
CCCAAGGCAC TTGGGACGTC CTGGACAGAC CGAGTCCCGG GAAGCCCCAG        150
CACTGCCGCT GCCACACTGC CCTGAGCCCA AATGGGCGAG TGAGAGGCCA        200

TAGCTGTCTG GC                                                 212

ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCA CTG GTG TTC   257
Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu
                  5                  10                  15

CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG   302
Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu
                 20                  25                  30

GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC   347
Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
                 35                  40                  45

CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC   392
Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
                 50                  55                  60

AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG   437
Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
                 65                  70                  75

GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC   482
Gly Gln Asp The Asp Cys Arg Glu Cys Gly Ser Gly Ser Phe Thr
                 80                  85                  90

GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC   527
Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys
                 95                 100                 105

CGA AAG GAA ATC GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC   572
Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
                110                 115                 120

CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT   617
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr
                125                 130                 135

TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC   662
Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu
                140                 145                 150

AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG   707
Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val
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                155                 160                 165

TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC  752
Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
                170                 175                 180

TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC AGG AAG TTG TGC  797
Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys
                185                 190                 195

CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC  842
Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
                200                 205                 210

ACA GTG CTG TTG CCC CTG GTC ATT TTC TTT GGT CTT TGC CTT TTA  887
Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu
                215                 220                 225

TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG  932
Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
                230                 235                 240

TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA  977
Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys
                245                 250                 255

GAG GGG GAG CTT GAA GGA ACT ACT ACT AAG CCC CTG GCC CCA AAC 1022
Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn
                260                 265                 270

CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC 1067
Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe
                275                 280                 285

AGT CCC GTG CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC 1112
Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr
                290                 295                 300

CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA GAG GTG GCA 1157
Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala
                305                 310                 315

CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC 1202
Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala
                320                 325                 330

TCC GAC CCC ATC CCC AAC CCC CTT CAG AAG TGG GAG GAC AGC GCC 1247
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Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys Trp Glu Asp Ser Ala
                335                 340                 345

CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAC 1292
His Lys Pro Gln Ser Leu Asp Thr Asp Asp Pro Ala Thr Leu Tyr
                350                 355                 360

GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAGGAATTC       1334
Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp
                365                 370
```

(67) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:371 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

```
Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu
  5                  10                  15
Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu
 20                  25                  30
Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
 35                  40                  45
Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
 50                  55                  60
Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
 65                  70                  75
Gly Gln Asp The Asp Cys Arg Glu Cys Gly Ser Gly Ser Phe Thr
 80                  85                  90
Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys
 95                 100                 105
Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
110                 115                 120
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr
125                 130                 135
Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu
140                 145                 150
Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val
155                 160                 165
Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
170                 175                 180
Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys
185                 190                 195
Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
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                200                 205                 210
Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu
                215                 220                 225
Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
                230                 235                 240
Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys
                245                 250                 255
Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn
                260                 265                 270
Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe
                275                 280                 285
Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr
                290                 295                 300
Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala
                305                 310                 315
Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala
                320                 325                 330
Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys Trp Glu Asp Ser Ala
                335                 340                 345
His Lys Pro Gln Ser Leu Asp Thr Asp Asp Pro Ala Thr Leu Tyr
                350                 355                 360
Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp
                365                 370
```

(68) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:6464 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

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TCGACATTGA TTATTGACTA GTTATTAATA GTAATCAATT ACGGGGTCAT   50
TAGTTCATAG CCCATATATG GAGTTCCGCG TTACATAACT TACGGTAAAT  100
GGCCCGCCTC GCTGACCGCC CAACGACCCC CGCCCATTGA CGTCAATAAT  150
GACGTATGTT CCCATAGTAA CGCCAATAGG GACTTTCCAT TGACGTCAAT  200
GGGTGGAGTA TTTACGGTAA ACTGCCCACT TGGCAGTACA TCAAGTGTAT  250
CATATGCCAA GTACGCCCCC TATTGACGTC AATGACGGTA AATGGCCCGC  300
CTGGCATTAT GCCCAGTACA TGACCTTATG GGACTTTCCT ACTIGGCAGT  350
ACATCTACGT ATTAGTCATC GCTATTACCA TGGTGATGCG GTTTTGGCAG  400
TACATCAATG GGCGTGGATA GCGGTTTGAC TCACGGGGAT TTCCAAGTCT  450
CCACCCCATT GACGTCAATG GGAGTTTGTT TTGGCACCAA AATCAACGGG  500
ACTTTCCAAA ATGTCGTAAC AACTCCGCCC CATTGACGCA AATGGGCGGT  550
AGGCGTGTAC GGTGGGAGGT CTATATAAGC AGAGCTCTCT GGCTAACTAG  600
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AGAACCCACT GCTTAACTGG CTTATCGAAA TTAATACGAC TCACTATAGG 650
GAGACCCAAG CTTCTGCAGG TCGACATCGA TGGATCCGGT ACCTCGAGCG 700
CGAATTCTCT AGAGGATCTT TGTGAAGGAA CCTTACTTCT GTGGTGTGAC 750
ATAATTGGAC AAACTACCTA CAGAGATTTA AAGCTCTAAG GTAAATATAA 800
AATTTTTAAG TGTATAATGT GTTAAACTAC TGATTCTAAT TGTTTGTGTA 850
TTTTAGATTC CAACCTATGG AACTGATGAA TGGGAGCAGT GGTGGAATGC 900
CTTTAATGAG GAAAACCTGT TTTGCTCAGA AGAAATGCCA TCTAGTGATG 950
ATGAGGCTAC TGCTGACTCT CAACATTCTA CTCCTCCAAA AAAGAAGAGA 1000
AAGGTAGAAG ACCCCAAGGA CTTTCCTTCA GAATTGCTAA GTTTTTTGAG 1050
TCATGCTGTG TTTAGTAATA GAACTCTTGC TTGCTTTGCT ATTTACACCA 1100
CAAAGGAAAA AGCTGCACTG CTATACAAGA AAATTATGGA AAAATATTTG 1150
ATGTATAGTG CCTTGACTAG AGATCATAAT CAGCCATACC ACATTTGTAG 1200
AGGTTTTACT TGCTTTAAAA AACCTCCCAC ACCTCCCCCT GAACCTGAAA 1250
CATAAAATGA ATGCAATTGT TGTTGTTAAC TTGTTTATTG CAGCTTATAA 1300
TGGTTACAAA TAAAGCAATA GCATCACAAA TTTCACAAAT AAAGCATTTT 1350
TTTCACTGCA TTCTAGTTGT GGTTTGTCCA AACTCATCAA TGTATCTTAT 1400
CATGTCTGGA TCAATTCTGA GAAACTAGCC TTAAAGACAG ACAGCTTTGT 1450
TCTAGTCAGC CAGGCAAGCA TATGTAAATA AAGTTCCTCA GGGAACTGAG 1500
GTTAAAAGAT GTATCCTGGA CCTGCCAGAC CTGGCCATTC ACGTAAACAG 1550
AAGATTCCGC CTCAAGTTCC GGTTAACAAC AGGAGGCAAC GAGATCTCAA 1600
ATCTATTACT TCTAATCGGG TAATTAAAAC CTTTCAACTA AAACACGGAC 1650
CCACGGATGT CACCCACTTT TCCTTCCCCG GCTCCGCCCT TCTCAGTACT 1700
CCCCACCATT AGGCTCGCTA CTCCACCTCC ACTTCCGGGC GCGACACCCA 1750
CGTGCCCTCT CCCACCCGAC GCTAACCCCG CCCCTGCCCG TCTGACCCCG 1800
CCCACCACCT GGCCCCGCCC CGTTGAGGAC AGAAGAAACC CCGGGCAGCC 1850
GCAGCCAAGG CGGACGGGTA GACGCTGGGG GCGCTGAGGA GTCGTCCTCT 1900
ACCTTCTCTG CTGGCTCGGT GGGGGACGCG GTGGATCTCA GGCTTCCGGA 1950
AGACTGGAAG AACCGGCTCA GAACCGCTTG TCTCCGCGGG GCTTGGGCGG 2000
CGGAAGAATG GCCGCTAGAC GCGGACTTGG TGCGAGGCAT CGCAGGATGC 2050
AGAAGAGCAA GCCCGCCGGG AGCGCGCGGC TGTACTACCC CGCGCCTGGA 2100
GCGGCCACGC CGGACTGGGC GGGGCCGGCC TGGTGGAGGC GGAGTCTGAC 2150
CTCGTGGAGG CGGGGCCTCT GATGTTCAAA TAGGATGCTA GGCTTGTTGA 2200
GGCGTGGCCT CCGATTCACA AGTGGGAAGC AGCGCCGGGC GACTGCAATT 2250
TCGCGCCAAA CTTGGGGGAA GCACAGCGTA CAGGCTGCCT AGGTGATCGC 2300
TGCTGCTGTC ATGGTTCGAC CGCTGAACTG CATCGTCGCC GTGTCCCAGA 2350
ATATGGGCAT CGGCAAGAAC GGAGACCTTC CCTGGCCAAT GCTCAGGTAC 2400
TGGCTGGATT GGGTTAGGGA AACCGAGGCG GTTCGCTGAA TCGGGTCGAG 2450
CACTTGGCGG AGACGCGCGG GCCAACTACT TAGGGACAGT CATGAGGGGT 2500
AGGCCCGCCG GCTGCTGCCC TTGCCCATGC CCGCGGTGAT CCCCATGCTG 2550
TGCCAGCCTT TGCCCAGAGG CGCTCTAGCT GGGAGCAAAG TCCGGTCACT 2600
GGGCAGCACC ACCCCCCGGA CTTGCATGGG TAGCCGCTGA GATGGAGCCT 2650
GAGCACACGT GACAGGGTCC CTGTTAACGC AGTGTTTCTC TAACTTTCAG 2700
GAACGAGTTC AAGTACTTCC AAAGAATGAC CACCACCTCC TCAGTGGAAG 2750
GTAAACAGAA CCTGGTGATT ATGGGCCGGA AAACCTGGTT CTCCATTCCT 2800
GAGAAGAATC GACCTTTAAA GGACAGAATT AATATAGTTC TCAGTAGAGA 2850
GCTCAAGGAA CCACCACAAG GAGCTCATTT TCTTGCCAAA AGTCTGGACC 2900

ATGCCTTAAA ACTTATTGAA CAACCAGAGT TAGCAGATAA AGTGGACATG 2950
GTTTGGATAG TTGGAGGCAG TTCCGTTTAC AAGGAAGCCA TGAATCAGCC 3000
AGGCCATCTC AGACTCTTTG TGACAAGGAT CATGCAGGAA TTTGAAAGTG 3050
ACACGTTCTT CCCAGAAATT GATTTGGAGA AATATAAACT TCTCCCAGAG 3100
TACCCAGGGG TCCTTTCTGA AGTCCAGGAG GAAAAAGGCA TCAAGTATAA 3150
ATTTGAAGTC TATGAGAAGA AAGGCTAACA GAAAGATACT TGCTGATTGA 3200
CTTCAAGTTC TACTGCTTTC CTCCTAAAAT TATGCATTTT TACAAGACCA 3250
TGGGACTTGT GTTGGCTTTA GATCCTGTGC ATCCTGGGCA ACTGTTGTAC 3300
TCTAAGCCAC TCCCCAAAGT CATGCCCCAG CCCCTGTATA ATTCTAAACA 3350
ATTAGAATTA TTTTCATTTT CATTAGTCTA ACCAGGTTAT ATTAAATATA 3400
CTTTAAGAAA CACCATTTGC CATAAAGTTC TCAATGCCCC TCCCATGCAG 3450
CCTCAAGTGG CTCCCCAGCA GATGCATAGG GTAGTGTGTG TACAAGAGAC 3500
CCCAAAGACA TAGAGCCCCT GAGAGCATGA GCTGATATGG GGGCTCATAG 3550
AGATAGGAGC TAGATGAATA AGTACAAAGG GCAGAAATGG GTTTTAACCA 3600
GCAGAGCTAG AACTCAGACT TTAAAGAAAA TTAGATCAAA GTAGAGACTG 3650
AATTATTCTG CACATCAGAC TCTGAGCAGA GTTCTGTTCA CTCAGACAGA 3700
AAATGGGTAA ATTGAGAGCT GGCTCCATTG TGCTCCTTAG AGATGGGAGC 3750
AGGTGGAGGA TTATATAAGG TCTGGAACAT TTAACTTCTC CGTTTCTCAT 3800
CTTCAGTGAG ATTCCAAGGG ATACTACAAT TCTGTGGAAT GTGTGTCAGT 3850
TAGGGTGTGG AAAGTCCCCA GGCTCCCCAG CAGGCAGAAG TATGCAAAGC 3900
ATGCATCTCA ATTAGTCAGC AACCAGGTGT GGAAAGTCCC CAGGCTCCCC 4000
AGCAGGCAGA AGTATGCAAA GCATGCATCT CAATTAGTCA GCAACCATAG 4050
TCCCGCCCCT AACTCCGCCC ATCCCGCCCC TAACTCCGCC CAGTTCCGCC 4100
CATTCTCCGC CCCATGGCTG ACTAATTTTT TTTATTTATG CAGAGGCCGA 4150
GGCGCCTCTG AGCTATTCCA GAAGTAGTGA GGAGGCTTTT TTGGAGGCCT 4200
AGGCTTITGC AAAAAAGCTA ATTCAGCCTG AATGGCGAAT GGGACGCGCC 4250
CTGTAGCGGC GCATTAAGCG CGGCGGGTGT GGTGGTTACG CGCAGCGTGA 4255
CCGCTACACT TGCCAGCGCC CTAGCGCCCG CTCCTTTCGC TTTCTTCCCT 4300
TCCTTTCTCG CCACGTTCGC CGGCTTTCCC CGTCAAGCTC TAAATCGGGG 4350
GCTCCCTTTA GGGTTCCGAT TTAGTGCTTT ACGGCACCTC GACCCCAAAA 4400
ACTTGATTAG GGTGATGGTT CACGTAGTGG GCCATCGCCC TGATAGACGG 4450
TTTTTCGCCC TTTGACGTTG GAGTCCACGT TCTTTAATAG TGGACTCTTG 4500
TTCCAAACTG GAACAACACT CAACCCTATC TCGGTCTATT CTTTTGATTT 4550
ATAAGGGATT TTGCCGATTT CGGCCTATTG GTTAAAAAAT GAGCTGATTT 4600
AACAAAAATT TAACGCGAAT TTTAACAAAA TATTAACGTT TACAATTTCA 4650
GGTGGCACTT TTCGGGGAAA TGTGCGCGGA ACCCCTATTT GTTTATTTTT 4700
CTAAATACAT TCAAATATGT ATCCGCTCAT GAGACAATAA CCCTGATAAA 4750
TGCTTCAATA ATATTGAAAA AGGAAGAGTA TGAGTATTCA ACATTTCCGT 4800
GTCGCCCTTA TTCCCTTTTT TGCGGCATTT TGCCTTCCTG TTTTTGCTCA 4850
CCCAGAAACG CTGGTGAAAG TAAAAGATGC TGAAGATCAG TTGGGTGCAC 4900
GAGTGGGTTA CATCGAACTG GATCTCAACA GCGGTAAGAT CCTTGAGAGT 4950
TTTCGCCCCG AAGAACGTTT TCCAATGATG AGCACTTTTA AAGTTCTGCT 5000
ATGTGGCGCG GTATTATCCC GTATTGACGC CGGGCAAGAG CAACTCGGTC 5050
GCCGCATACA CTATTCTCAG AATGACTTGG TTGAGTACTC ACCAGTCACA 5100
GAAAAGCATC TTACGGATGG CATGACAGTA AGAGKATTAT GCAGTGCTGC 5150
CATAACCATG AGTGATAACA CTGCGGCCAA CTTACTTCTG ACAACGATCG 5200
GAGGACCGAA GGAGCTAACC GCTTTTTTGC ACAACATGGG GGATCATGTA 5250

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ACTCGCCTTG ATCGTTGGGA ACCGGAGCTG AATGAAGCCA TACCAAACGA 5300
CGAGCGTGAC ACCACGATGC CTGTAGCAAT GGCAACAACG TTGCGCAAAC 5350
TATTAACTGG CGAACTACTT ACTCTAGCTT CCCGGCAACA ATTAATAGAC 5400
TGGATGGAGG CGGATAAAGT TGCAGGACCA CTTCTGCGCT CGGCCCTTCC 5450
GGCTGGCTGG TTTATTGCTG ATAAATCTGG AGCCGGTGAG CGTGGGTCTC 5500
GCGGTATCAT TGCAGCACTG GGGCCAGATG GTAAGCCCTC CCGTATCGTA 5550
GTTATCTACA CGACGGGGAG TCAGGCAACT ATGGATGAAC GAAATAGACA 5600
GATCGCTGAG ATAGGTGCCT CACTGATTAA GCATTGGTAA CTGTCAGACC 5650
AAGTTTACTC ATATATACTT TAGATTGATT TAAAACTTCA TTTTTAATTT 5700
AAAAGGATCT AGGTGAAGAT CCTTTTTGAT AATCTCATGA CCAAAATCCC 5750
TTAACGTGAG TTTTCGTTCC ACTGAGCGTC AGACCCCGTA GAAAAGATCA 5800
AAGGATCTTC TTGAGATCCT TTTTTTCTGC GCGTAATCTG CTGCTTGCAA 5850
ACAAAAAAAC CACCGCTACC AGCGGTGGTT TGTTTGCCGG ATCAAGAGCT 5900
ACCAACTCTT TTTCCGAAGG TAACTGGCTT CAGCAGAGCG CAGATACCAA 6000
ATACTGTCCT TCTAGTGTAG CCGTAGTTAG GCCACCACTT CAAGAACTCT 6050
GTAGCACCGC CTACATACCT CGCTCTGCTA ATCCTGTTAC CAGTGGCTGC 6100
TGCCAGTGGC GATAAGTCGT GTCTTACCGG GTTGGACTCA AGACGATAGT 6150
TACCGGATAA GGCGCAGCGG TCGGGCTGAA CGGGGGGTTC GTGCACACAG 6200
CCCAGCTTGG AGCGAACGAC CTACACCGAA CTGAGATACC TACAGCGTGA 6250
GCATTGAGAA AGCGCCACGC TTCCCGAAGG GAGAAAGGCG GACAGGTATC 6300
CGGTLAGCGG CAGGGTCGGA ACAGGAGAGC GCACGAGGGA GCTTCCAGGG 6350
GGAAACGCCT GGTATCTTTA TAGTCCTGTC GGGTTTCGCC ACCTCTGACT 6400
TGAGCGTCGA TTTTTGTGAT GCTCGTCAGG GGGGCGGAGC CTATGGAAAA 6450
ACGCCAGCAA CGCC                                        6464
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(69) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:2173 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

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GAATTCCTTT TCTCCGAGTT TTCTGAACTC TGGCTCATGA TCGGGCTTAC         50
TGGATACGAG AATCCTGGAG GACCGTACCC TGATTTCCAT CTACCTCTGA        100
CTTTGAGCCT TTCTAACCCG GGGCTCACGC TGCCAACACC CGGGCCACCT        150
GGTCCGATCG TCTTACTTCA TTCACCAGCG TTGCCAATTG CTGCCCTGTC        200
CCCAGCCCCA ATGGGGGAGT GAGAGAGGCC ACTGCCGGCC GGAC             244

ATG GGT CTC CCC ATC GTG CCT GGC CTG CTG CTG TCA CTG GTG CTC   289
Met Gly Leu Pro Ile Val Pro Gly Leu Leu Leu Ser Leu Val Leu
                  5                  10                  15

CTG GCT CTG CTG ATG GGG ATA CAC CCA TCA GGG GTC ACC GGA CTG   334
Leu Ala Leu Leu Met Gly Ile His Pro Ser Gly Val Thr Gly Leu
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                  20                  25                  30

GTT CCT TCT CTT GGT GAC CGG GAG AAG AGG GAT AAT TTG TGT CCC  379
Val Pro Ser Leu Gly Asp Ara Glu Lys Arg Asp Asn Leu Cys Pro
                  35                  40                  45

CAG GGA AAG TAT GCC CAT CCA AAG AAT AAT TCC ATC TGC TGC ACC  424
Gln Gly Lys Tyr Ala His Pro Lys Asn Asn Ser Ile Cys Cvs Thr
                  50                  55                  60

AAG TGC CAC AAA GGA ACC TAC TTG GTG AGT GAC TGT CCA AGC CCA  469
Lys Cys His Lys Gly Thr Tyr Leu Val Ser Asp Cys Pro Ser Pro
                  65                  70                  75

GGG CAG GAA ACA GTC TGC GAG CTC TCT CAT AAA GGC ACC TTT ACA  514
Gly Gln Glu Thr Val Cys Glu Leu Ser His Lys Gly Thr Phe Thr
                  80                  85                  90

GCT TCG CAG AAC CAC GTC AGA CAG TGT CTC AGT TGC AAG ACA TGT  559
Ala Ser Gln Asn His Val Arg Gln Cys Leu Ser Cys Lys Thr Cys
                  95                 100                 105

CGG AAA GAA ATG TTC CAG GTG GAG ATT TCT CCT TGC AAA GCT GAC  604
Arg Lys Glu Yet Phe Gln Val Glu Ile Ser Pro Cys Lys Ala Asp
                 110                 115                 120

ATG GAC ACC GTG TGT GGC TGC AAG PAG AkC CAA TTC CAG CGC TAC  649
Met Asp Thr Val Cys Gly Cys Lys Lys Asn Gln Phe Gln Arg Tyr
                 125                 130                 135

CTG AGT GAG ACG CAT TTC CAG TGT GTG GAC TGC AGC CCC TGC TTC  694
Leu Ser Glu Thr His Phe Gln Cys Val Asp Cys Ser Pro Cys Phe
                 140                 145                 150

AAT GGC ACC GTG ACA ATC CCC TGT AAG GAG AAA CAG AAC ACC GTG  739
Asn Gly Thr Val Thr Ile Pro Cys Lys Glu Lys Gln Asn Thr Val
                 155                 160                 165

TGT AAC TGC CAC GCA GGA TTC TTT CTA AGC GGA AAT GAG TGC ACC  784
Cys Asn Cys His Ala Gly Phe Phe Leu Ser Gly Asn Glu Cys Thr
                 170                 175                 180

CCT TGC AGC CAC TGC AAG AAA AAT CAG GAA TGT ATG AAG CTG TGC  829
Pro Cys Ser His Cys Lys Lys Asn Gln Glu Cys Met Lys Leu Cys
                 185                 190                 195

CTA CCT CCA GTT GCA AAT GTC ACA AAC CCC CAG GAC TCA GGT ACT  874
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Leu Pro Pro Val Ala Asn Val Thr Asn Pro Gln Asp Ser Gly Thr
                200                 205                 210

GCC GIC CTG TTG CCT CTG GTT ATC TTC CTA GGT CTT TGC CTT TTA  919
Ala Val Leu Leu Pro Leu Val Ile Phe Leu Gly Leu Cys Leu Leu
                215                 220                 225

TTC TTT ATC TGC ATC AGT CTA CTG TGC CGA TAT CCC CAG TGG AGG  964
Phe Phe Ile Cys Ile Ser Leu Leu Cys Arg Tyr Pro Gln Trp Arg
                230                 235                 240

CCC AGG GTC TAC TCC ATC ATT TGT AGG GAT TCA GCT CCT GTC AAA 1009
Pro Arg Val Tyr Ser Ile Ile Cys Arg Asp Ser Ala Pro Val Lys
                245                 250                 255

GAG GTG GAG GGT GAA GGA ATT GTT ACT AAG CCC CTA ACT CCA GCC 1054
Glu Val Glu Gly Glu Gly Ile Val Thr Lys Pro Leu Thr Pro Ala
                260                 265                 270

TCT ATC CCA GCC TTC AGC CCC AAC CCC GGC TTC AAC CCC ACT CTG 1099
Ser Ile Pro Ala Phe Ser Pro Asn Pro Gly Phe Asn Pro Thr Leu
                275                 280                 285

GGC TTC AGC ACC ACC CCA CGC TTC AGT CAT CCT GTC TCC AGT ACC 1144
Gly Phe Ser Thr Thr Pro Arg Phe Ser His Pro Val Ser Ser Thr
                290                 295                 300

CCC ATC AGC CCC GTC TTC GGT CCT AGT AAC TGG CAC AAC TTC GTG 1189
Pro Ile Ser Pro Val Phe Gly Pro Ser Asn Trp His Asn Phe Val
                305                 310                 315

CCA CCT GTA AGA GAG GTG GTC CCA ACC CAG GGT GCT GAC CCT CTC 1234
Pro Pro Val Arg Glu Val Val Pro Thr Gln Gly Ala Asp Pro Leu
                320                 325                 330

CTC TAC GGA TCC CTC AAC CCT GTG CCA ATC CCC GCC CCT GTT CGG 1279
Leu Tyr Gly Ser Leu Asn Pro Val Pro Ile Pro Ala Pro Val Arg
                335                 340                 345

AAA TGG GAA GAC GTC GTC GCG GCC CAG CCA CAA CGG CTT GAC ACT 1324
Lys Trp Glu Asp Val Val Ala Ala Gln Pro Gln Arg Leu Asp Thr
                350                 355                 360

GCA GAC CCT GCG ATG CTG TAT GCT GTG GTG GAT GGC GTG CCT CCG 1369
Ala Asp Pro Ala Met Leu Tyr Ala Val Val Asp Gly Val Pro Pro
                365                 370                 375
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ACA CGC TGG AAG GAG TTC ATG CGG CTC CTG GGG CTG AGC GAG CAC 1414
Thr Arg Trp Lys Glu Phe Met Arg Leu Leu Gly Leu Ser Glu His
                380                 385                 390

GAG ATC GAG CGG TTG GAG CTG CAG AAC GGG CGT TGC CTC CGC GAG 1459
Glu Ile Glu Arc Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu
                395                 400                 405

GCT CAT TAC AGC ATG CTG GAA GCC TGG CGG CGC CGC ACA CCG CGA 1504
Ala His Tyr Ser Met Leu Glu Ala Trp Arg Arg Arg Thr Pro Arg
                410                 415                 420

CAC GAG GCC ACG CTG GAC GTA GTG GGC CGC GTG CTT TGC GAC ATG 1549
His Glu Ala Thr Leu Asp Val Val Gly Arg Val Leu Cys Asp Met
                425                 430                 435

AAC CTG CGT GGC TGC CTG GAG AAC ATC CGC GAG ACT CTA GAA AGC 1594
Asn Leu Arg Gly Cys Leu Glu Asn Ile Arg Glu Thr Leu Glu Ser
                440                 445                 450

CCT GCC CAC TCG TCC ACG ACC CAC CTC CCG CGA TAAGGCCACA      1637
Pro Ala His Ser Ser Thr Thr His Leu Pro Arg
                455                 460

CCCCCACCTC AGGAACGGGA CTCGAAGGAC CATCCTGCTA GATGCCCTGC      1687
TTCCCTGTGA ACCTCCTCTT TGGTCCTCTA GGGGGCAGGC TCGATCTGGC      1737
AGGCTCGATC TGGCAGCCAC TTCCTTGGTG CTACCGACTT GGTGTACATA      1787
GCTTTTCCCA GCTGCCGAGG ACAGCCTGTG CCAGCCACTT GTGCATGGCA      1837
GGGAAGTGTG CCATCTGCTC CCAGACAGCT GAGGGTGCCA AAAGCCAGGA      1887
GAGGTGATTG TGGAGAAAAA GCACAATCTA TCTGATACCC ACTTGGGATG      1937
CAAGGACCCA AACAAAGCTT CTCAGGGCCT CCTCAGTTGA TTTCTGGGCC      1987
CTTTTCACAG TAGATAAAAC AGTCTTTGTA TTGATTATAT CACACTAATG      2037
GATGAACGGT TGAACTCCCT AAGGTAGGGG CAAGCACAGA ACAGTGGGGT      2087
CTCCAGCTGG AGCCCCCGAC TCTTGTAAAT ACACTAAAAA TCTAAAAGTG      2137
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAG GAATTC                     2173
```

(70) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:461 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

```
Met Gly Leu Pro Ile Val Pro Gly Leu Leu Leu Ser Leu Val Leu
                  5                  10                  15
Leu Ala Leu Leu Met Gly Ile His Pro Ser Gly Val Thr Gly Leu
```

```
                  20                  25                  30
Val Pro Ser Leu Gly Asp Ara Glu Lys Arg Asp Asn Leu Cys Pro
                  35                  40                  45

Gln Gly Lys Tyr Ala His Pro Lys Asn Asn Ser Ile Cys Cvs Thr
                  50                  55                  60
Lys Cys His Lys Gly Thr Tyr Leu Val Ser Asp Cys Pro Ser Pro
                  65                  70                  75
Gly Gln Glu Thr Val Cys Glu Leu Ser His Lys Gly Thr Phe Thr
                  80                  85                  90
Ala Ser Gln Asn His Val Arg Gln Cys Leu Ser Cys Lys Thr Cys
                  95                 100                 105
Arg Lys Glu Yet Phe Gln Val Glu Ile Ser Pro Cys Lys Ala Asp
                 110                 115                 120
Met Asp Thr Val Cys Gly Cys Lys Lys Asn Gln Phe Gln Arg Tyr
                 125                 130                 135
Leu Ser Glu Thr His Phe Gln Cys Val Asp Cys Ser Pro Cys Phe
                 140                 145                 150
Asn Gly Thr Val Thr Ile Pro Cys Lys Glu Lys Gln Asn Thr Val
                 155                 160                 165
Cys Asn Cys His Ala Gly Phe Phe Leu Ser Gly Asn Glu Cys Thr
                 170                 175                 180
Pro Cys Ser His Cys Lys Lys Asn Gln Glu Cys Met Lys Leu Cys
                 185                 190                 195
Leu Pro Pro Val Ala Asn Val Thr Asn Pro Gln Asp Ser Gly Thr
                 200                 205                 210
Ala Val Leu Leu Pro Leu Val Ile Phe Leu Gly Leu Cys Leu Leu
                 215                 220                 225
Phe Phe Ile Cys Ile Ser Leu Leu Cys Arg Tyr Pro Gln Trp Arg
                 230                 235                 240
Pro Arg Val Tyr Ser Ile Ile Cys Arg Asp Ser Ala Pro Val Lys
                 245                 250                 255
Glu Val Glu Gly Glu Gly Ile Val Thr Lys Pro Leu Thr Pro Ala
                 260                 265                 270
Ser Ile Pro Ala Phe Ser Pro Asn Pro Gly Phe Asn Pro Thr Leu
                 275                 280                 285
Gly Phe Ser Thr Thr Pro Arg Phe Ser His Pro Val Ser Ser Thr
                 290                 295                 300
Pro Ile Ser Pro Val Phe Gly Pro Ser Asn Trp His Asn Phe Val
                 305                 310                 315
Pro Pro Val Arg Glu Val Val Pro Thr Gln Gly Ala Asp Pro Leu
                 320                 325                 330
Leu Tyr Gly Ser Leu Asn Pro Val Pro Ile Pro Ala Pro Val Arg
                 335                 340                 345
Lys Trp Glu Asp Val Val Ala Ala Gln Pro Gln Arg Leu Asp Thr
                 350                 355                 360
Ala Asp Pro Ala Met Leu Tyr Ala Val Val Asp Gly Val Pro Pro
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                365                 370                 375
Thr Arg Trp Lys Glu Phe Met Arg Leu Leu Gly Leu Ser Glu His
                380                 385                 390
Glu Ile Glu Arc Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu
                395                 400                 405
Ala His Tyr Ser Met Leu Glu Ala Trp Arg Arg Arg Thr Pro Arg
                410                 415                 420
His Glu Ala Thr Leu Asp Val Val Gly Arg Val Leu Cys Asp Met
                425                 430                 435
Asn Leu Arg Gly Cys Leu Glu Asn Ile Arg Glu Thr Leu Glu Ser
                440                 445                 450
Pro Ala His Ser Ser Thr Thr His Leu Pro Arg
                455                 460
```

(71) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:1232 bases
(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

```
GAATTCTCTG GACTGAGGCT CCAGTTCTGG CCTTTGGGGT TCAAGATCAC         50
TGGGACCAGG CCGTGATCTC TATGCCCGAG TCTCAACCCT CAACTGTCAC        100
CCCAAGGCAC TTGGGACGTC CTGGACAGAC CGAGTCCCGG GAAGCCCCAG        150
CACTGCCGCT GCCACACTGC CCTGAGCCCA GATGGGGGAG TGAGAGGCCA        200
TAGCTGTCTG GC                                                212

ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCA CTG GTG CTC   257
Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu
  5                  10                  15

CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG   302
Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu
                20                  25                  30

GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC   347
Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
                35                  40                  45

CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC   392
Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
                50                  55                  60

AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG   437
```

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Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
                 65                  70                  75

GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC  482
Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr
                 80                  85                  90

GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC  527
Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys
                 95                 100                 105

CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC  572
Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
                110                 115                 120

CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT  617
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr

                125                 130                 135

TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC  662
Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu
                140                 145                 150

AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG  707
Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val
                155                 160                 165

TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC  752
Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
                170                 175                 180

TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC  797
Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys
                185                 190                 195

CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC  842
Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
                200                 205                 210

ACA GTG CTG TTG CCC CTG GTC ATT TTC TTT GGT CTT TGC CTT TTA  887
Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu
                215                 220                 225

TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG  932
Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
                230                 235                 240
```

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TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA  977
Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys
                245                 250                 255

GAG GGG GAG CTT GAA GGA ACT ACT ACT AAG CCC CTG GCC CCA AAC 1022
Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn
                260                 265                 270

CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC 1067
Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe
                275                 280                 285

AGT CCC GTG CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC 1112
Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr
                290                 295                 300

CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA GAG GTG GCA 1157
Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala
                305                 310                 315

CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC 1202
Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala
                320                 325                 330

TCC GAC CCC ATC CCC AAC CCC CTT CAG AAG                     1232

Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys

                335                 340
```

(72) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:340 amino acids
(B) TYPE:polypeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

```
Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu
  5                  10                  15
Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu
 20                  25                  30
Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
 35                  40                  45

Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
```

```
                  50                  55                  60
Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
                  65                  70                  75
Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr
                  80                  85                  90
Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys
                  95                 100                 105
Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
                 110                 115                 120
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr

                 125                 130                 135
Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu
                 140                 145                 150
Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val
                 155                 160                 165
Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
                 170                 175                 180
Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys
                 185                 190                 195
Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
                 200                 205                 210
Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu
                 215                 220                 225
Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
                 230                 235                 240
Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys
                 245                 250                 255
Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn
                 260                 265                 270
Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe
                 275                 280                 285
Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr
                 290                 295                 300
Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala
                 305                 310                 315
Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala
                 320                 325                 330
Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys

                 335                 340
```

(73) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH:19 bases

(B) TYPE:nucleic acid
(C) STRANDEDNESS:single
(D) TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GTACTTGAAC TCGTTCCTG 19

Appendix D

D 18.1

EP 90 12 4133.1

STATE OF ISRAEL

This is to certify that annexed hereto is a true copy of the documents as originally deposited with the patent application particulars of which are specified on the first page of the annex.

זאת לתעודה כי רצופים בזה העתקים נאמנים של המסמכים שהופקדו לכתחילה עם הבקשה לפטנט לפי הפרטים הרשומים בעמוד הראשון של הנספח.

This _____ היום

רשם הפטנטים
Registrar of Patents

לשימוש הלשכה
For Office Use

092697	מספר: Number
13 -12- 1989	תאריך: Date
	הוקדם/נדחה Ante/Post-dated

חוק הפטנטים, תשכ"ן – 1967
PATENT LAW, 5727 - 1967

בקשה לפטנט
Application for Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
I (Name and address of applicant, and in case of body corporate-place of incorporation)

Yeda Research and Development Co. Ltd.
A company registered under the laws of Israel

P.O. Box 95, Rehovot, Israel

ידע חברה למחקר ופיתוח בע"מ
חברה רשומה בישראל
ת.ד. 95
רחובות

שטרה הוא Assignment by the inventors
of an invention the title of which is

העברה מהממציאים
בעל זכות מכח
Owner, by virtue of

(בעברית) (Hebrew) שיבוט מולקולרי של חלבון הקושר TNF

(באנגלית) (English) Molecular cloning of TNF Binding Protein

hereby apply for a patent to be granted to me in respect thereof.

מבקש בזאת כי יתן לי עליה פטנט

• בקשת חלוקה – Application of Division	• בקשת פטנט פטף – Application for Patent Addition	• דרישת דין קדימה Priority Claim		
מבקשת פטנט from Application	• לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האגוד Convention Country
No. מס	No. מס			
dated מיום	dated מיום			
• יפוי כח: כללי / מיוחד – רצוף בזה / עוד יוגש P.O.A.: general/individual-attached/to be filed later.				
הוגש בענין filed in case				

תמען לצורת מסמכים בישראל
Address for Service in Israel
Paulina Ben-Ami, Patent Attorney,
Inter-Lab Ltd., Kiryat Weizmann,
Ness-Ziona 76110, Israel

חתימת המבקש
Signature of Applicant

For the Applicant:

Paulina Ben-Ami

היום 11 בחודש דצמבר שנת 1989
This 11 day of December of the year 1989

לשימוש הלשכה
For Office Use

שיבוט מולקולרי של חלבון הקושר TNF

Molecular cloning of TNF Binding Protein

Yeda Research and
Development Co. Ltd.
T/811

The invention relates to Tumor Necrosis Factor (TNF) Binding Protein I and more particularly, to the cloning of gene coding for said protein.

Patent Application No. 83878 of the same applicant discloses a new protein found in urine and capable of inhibiting the binding of TNF to its receptors and the cytotoxic effect of TNF. This protein is now referred to hereinafter as TNF Binding Protein I or TBP-I.

The process for the extraction and purification of TBP-I in the above mentioned patent application comprises the following steps:

(a) recovering the crude protein fraction from a dyalized concentrate of human urine;

(b) subjecting said crude protein fraction of step (a) to ion exchange chromatography to obtain partially purified active fractions of the TNF Binding Protein defined by its ability to inhibit both the binding of TNF to its receptors and the cytotoxic effect of TNF;

(c) applying said partially purified active fractions of the TNF Binding Protein from step (b) to reversed phase high pressure liquid chromatography (HPLC) to obtain substantially purified active fractions of the TNF Binding Protein defined by its ability to inhibit both the binding of TNF to its receptors and the cytotoxic effect of TNF; and

(d) recovering the substantially purified protein of step (c), said protein having a molecular weight of about 26-28 Kda on SDS PAGE under reducing conditions, moving as a single peak on reversed phase HPLC and having the ability to inhibit both the binding of TNF to its receptors and the cytotoxic effect of TNF.

The purified TBP-I was sequenced and shown to contain at the N-terminus the following amino acid sequence:

1				5				10				15
Asp	Ser	Val	Cys	Pro	Gln	Gly	Lys	Tyr	Ile	His	Pro	Gln	Asn	Asn	Ser

The invention relates to oligonucleotide probes to the cDNA coding for a protein comprising the amino acid sequence of TBP-1. The probes were synthesized by known methods on the basis of the above amino acid sequence of the N-terminus of TBP-I.

The invention also relates to a DNA molecule comprising a recombinant DNA molecule or a cDNA molecule coding for a protein comprising the amino acid sequence of TBP-I or a protein substantially homologous therewith. Within the scope of the invention are DNA molecules encoding said homologous proteins having the same biological activities of TBP-I.

In a preferred embodiment, the DNA molecule is a cDNA molecule picked up from a human cDNA library, in particular a colon cDNA library. Illustrated in Figure 1 is a partial restriction map of an insert of about 1.0 Kb of such a cDNA molecule obtained in agarose gel and herein designated C2. Figure 2 illustrates a partial nucleotide sequence of said C2 insert and also a partial translated amino acid sequence comprising the NH_2-terminal amino acid sequence of TBP-I encoded thereby. Figure 3 illustrates another partial nucleotide sequence of the C2 insert starting from nucleotide 342. Figure 4 shows a possible nucleotide sequence of the whole insert, that seems to have 965 nucleotides.

The invention further comprises cloning of said cDNA molecule

into a replicable plasmid vector and transformation of a bacterium, e.g., competent E.coli TG1 therewith.

In another aspect, the invention comprises the isolation of mRNA coding for a protein comprising the amino acid sequence of TBP-I by extraction from cells and its detection by hybridization with the cDNA of the invention.

Once the mRNA is obtained in a purified form, the cDNA coding for a protein comprising the amino acid sequence of TBP-I can be obtained by contacting the mRNA with reverse transcriptase for a time and under conditions sufficient to form said cDNA. This cDNA may be converted to double stranded cDNA by known techniques.

Probes may be prepared from the cDNA sequences of the invention and used for isolation of the genomic DNA coding for a protein comprising the amino acid sequence of TBP-I by known methods, e.g. by colony hybridization techniques under stringent conditions.

The DNA of positive clones are then inserted into appropriately constructed expression vectors by techniques well known in the art. Double-stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques. DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing a desired protein, an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding for the desired protein in such a way as to permit gene expression and production of the protein. The gene must be preceded by a promoter in order to be transcribed. There are a

variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

The DNA molecule comprising the nucleotide sequence coding for a protein comprising the amino acid sequence of TBP-I preceded by a nucleotide sequence of a signal peptide and the operably linked transcriptional and translational regulatory signals is inserted into a vector which is capable of integrating the desired gene sequences into the host cell chromosome. The cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector.

In a preferred embodiment, the introduced DNA molecule will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Factors of importance in selecting a particular plasmid or viral vector include the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate precipitation, direct microinjection, etc.

Host cells to be used in this invention may be either prokaryotic or eukaryotic. Preferred prokaryotic hosts include bacteria, such as E.coli. Under such conditions, the protein will

not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

Preferred eukaryotic hosts are mammalian cells, e.g., human, monkey, mouse and chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Also yeast and insect cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e. pre-peptides).

After the introduction of the vector, the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired protein or a fragment thereof. The expressed protein is then isolated and purified by any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like.

The invention will be illustrated by the following examples:

Example 1

Preparation of oligonucleotide probes

Oligonucleotide probes to the cDNA of TBP-I were designed on the basis of the NH_2-terminal amino acid sequence of the protein. Three mixtures of the synthetic oligonucleotides shown below and designated 1008, 1009, 1010 were used.

Probe 1008 is a mixture of 64 different 26-mers, in which deoxyinosine was introduced wherever the number of possible alternative codons for the amino acid exceeded 3. The two other mixtures are 17-mers; the first, probe 1009, is a mixture of 128 different oligonucleotides and the second, probe 1010, of 196 different oligonucleotides. Each of these two latter mixtures corresponds to part of the amino acid sequence coded-for by 1008. The nucleotide sequence of these two mixtures overlap each other.

1008	GGI	GTC	CCI	TTC	ATA	TAA	GTA	GGI	GT
		T		T	G	G	G		
							T		
1009	GGA	GTC	CCA	TTC	ATA	TA			
	C	T	C	T	G				
	T		G						
	G		T						
1010				TTC	ATA	TAA	GTA	GGA	GT
				T	G	G	G	C	
						T		G	
								T	

Example 2

Isolation of cDNA clones

cDNA clones comprising a nucleotide sequence coding for a protein comprising the amino acid sequence of TBP-I were isolated from a human cDNA colon library with the aid of the oligonucleotide probes of Example I as follows:

Four cDNA libraries constructed in lambda gt11 (Clontech Laboratories, Inc., U.S.A.) derived from the mRNA of human liver, human placenta, human colon and of HeLa cells were screened with the aid of the 1008 probes of Example 1. The liver, placenta and HeLa cDNA libraries were oligo dT primed, while the colon cDNA library was randomly primed. In each screening, 5×10^6 phages were adsorbed to

Escherichia coli, strain Y1088, plated at a density of 40,000 p.f.u/15 cm petri dish and grown at 37°C for 18 hours. Nitrocellulose filters were overlaid in duplicates on the plates, then immersed in DNA-denaturing solution, transferred further to a neutralizing solution, and then dried in vacuum at 80°C and prehybridized to allow non-specific sites to be saturated with unlabelled DNA. The 1008 probes were ^{32}P-end-labelled, using the T4 polynucleotide kinase and applied to the filters in a solution containing 6 SSC (1 SSC corresponds to 0.15M NaCl and 0.015M sodium citrate), 10 "Denhardt's solution" (a mixture of Ficoll, polyvinylpyrrolidone and bovine serum albumin (Pentax Fraction V) in water, according to T. Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, N.Y., 1982 p. 448), 0.5% SDS (sodium dodecyl sulfate), and 100 µg/ml salmon sperm DNA. Hybridization was carried out for 18 hours at 50°C with the colon library and at 42°C with the other libraries. Unbound labelled probe was washed with a solution containing 3SSC at 25°C and then twice again either at 42°C or, for the colon library, at 50°C. Positive clones, identified by exposure of the filters to autoradiography, were picked up, purified and checked for hybridization to the 1009 and 1010 probes under those same conditions which were applied for the screening with the 1008 probes, except that the temperature of hybridization and washing was 30°C.

The results of the screening are summarized in Table I. Clones which hybridized with all three probes could be detected only in the placenta and colon libraries. In further analysis of the nucleotide sequence, only the clones picked up from the colon library (the only library which was randomly primed) were found indeed to code for TBP-I. These clones were designated C1, C2, C3, and C4.

Table I: Libraries screened for the TBP-I cDNA and the clones which were isolated

Vector	Library	Clone name	Temp. of hybridization 1008 C°	1009 C°	1010 C°
λgt11 cDNA oligo dT primed					
	Liver	-	-	-	-
	HeLa	-	-	-	-
	Placenta	17	50°	-	-
		19	50°	30°	30°
		131	60°	30°	30°
		133	50°	30°	30°
		152	50°	-	-
λgt11 cDNA randomly primed					
	Colon*	C1	60°	30°	30°
		C2	60°	30°	30°
		C3	60°	30°	30°
		C4	60°	30°	30°

*normal tissue around colon cancer

Example 3

Characterization of the isolated clones from the human colon cDNA library

The purified lambda gt11 DNA containing positive cDNA clones were digested with EcoR1 and size-fractionated on 1% agarose gel. Two of the clones had an insert size of about 1.0 Kb, the third was of 0.9 Kb and the fourth had two inserts of 0.9 and 0.8 Kb. Cross-hybridization among the four clones was tested by Southern blotting. The results are summarized in Table II. Clones C2 and C3 were found to contain the same 1.0 Kb insert. The restriction map of this insert is shown in Figure 1. Clone C5 contains two inserts, of 0.9 and 0.8 Kb: the 0.9 Kb insert constitutes part of the insert of C2, while the 0.8 Kb insert seems to be unrelated. Clone C1 also contains

a 0.9 Kb insert which constitutes part of the insert of C2.

Table II: Insert sizes and interrelationships in the various cDNA clones for TBP-I

cDNA clone	Insert size (Kb)	Cross hybridization: with the C1 insert	with the C2 insert	with the EcoRI-PstI 165 nucleotide fragment of C2
C1	0.9	++	+	++
C2	1.0	+	++	++
C3	1.0	+	++	++
C5	0.9, 0.8	+	++	++

The 1.0 Kb EcoRI insert of the C2 clone was subcloned in a Bluescript plasmid vector of Stratagene Cloning System (San Diego, Cal.) and E. coli TG1 competent bacteria were transformed therewith. The transformed bacteria were deposited with the Collection Nationale de Cultures de Microorganismes (C.N.C.M.), of the Institut Pasteur, Paris, France, on December 6, 1989 under the Budapest Treaty, and it was assigned the deposit number CNCM I-917.

Example 4

Screening of an oligo dT primed (human placenta) cDNA library with the DNA probes

To isolate sequences extending 3' to the insert of clone C2, 0.5×10^6 recombinants from human placenta cDNA library in lambda gt11 were screened for hybridization with the aid of a probe prepared from the 125-nucleotide PstI-PstI fragment of clone C2 (see Fig. 1) which was labelled with the multiprime DNA labelling systems kit

(Amersham). The technique is based on the use of random sequence hexanucleotide to prime DNA synthesis on denatured template DNA at numerous sites along its length.

Phages were adsorbed to E.coli strain Y1088, plated at a density of 40,000 p.f.u/15 cm petri dish and grown at 37°C overnight. Two sets of nitrocellulose filters were overlaid and immersed in a tray containing DNA-denaturing solution. The filters were washed, fixed, neutralized, dried at 80°C under vacuum and prehybridized to allow non-specific sites to be saturated by unlabelled DNA. Then the filters were hybridized with the ^{32}P-labelled probe overnight at 65°C. Unbound label was washed first in a solution containing 1SSC and 0.1% SDS (twice at 25°C and then twice again at 65°C) and then at 65°C in a solution containing 0.1 SSC and 0.1% SDS. Filters were autoradiographed. Thirteen positive clones were obtained and picked up. After purification, these clones were tested for hybridization with a probe constructed from the C2 insert from which the above EcoRI-PstI insert was deleted. Four clones were found to hybridize. DNA from these positive clones was isolated after purifying the phages by centrifugation in a CsCl solution. Their inserts were excised by cutting with EcoRI and their sizes were estimated by electrophoresis on 1% agarose gel. The phage containing the largest fragment of about 2.0 Kb was subjected to further analysis. Its insert was subcloned in a Bluescript plasmid vector and E.coli TG 1 competent bacteria were transformed therewith.

Example 5

Determination of the nucleotide sequence in the cloned DNA

DNA of the 1.0 Kb EcoRI insert of the C2 clone propagated in the

Bluescript plasmid vector was subjected to partial degradation to various extents using the Erase-a-Base Progema kit. DNA of plasmids containing the insert at various degrees of degradation was denatured and subjected to sequencing as described by Hattoni and Sakaki (Anal. Biochem. 152, 232-238) with the aid of the Sequenase Kit (USB).

The insert was shown to have 965 nucleotides. Figure 2 shows a partial nucleotide sequence of the C2 insert and the deduced amino acid sequence which it codes for. Within this sequence the NH_2-terminal amino acid sequence of TBP-I, on the basis of which the cDNA had been cloned, can be detected (underlined). Figure 3 shows the 343-965 nucleotide sequence of the C2 insert and Figure 4 shows a possible total nucleotide sequence of the C2 insert.

Example 6

Detection and sizing of the mRNA for TBP-I by Northern blot analysis

Total RNA was extracted from cells of the U937 and HT29 lines by the 'hot phenol' method according to T. Maniatis et al., op. cit., p. 194. Samples of 50 and 25µg RNA were analyzed by electrophoresis on 1.5% agarose gel in the presence of 2.2% formaldehyde followed by blotting to charged nylon filter. The EcoRI insert of the C2 clone was ^{32}P-labeled with the use of the multiprime DNA labelling systems kits and hybridized to the charged nylon blot (42°C in 50% formamide). As shown in Figure 5 in both cells the cDNA was found to hybridize to mRNA of just a single size, of about 19S-21S and having about 2500 nucleotides (right stronger dot - 50 µg RNA, left weaker dot - 25 µg RNA).

FIGURE 1

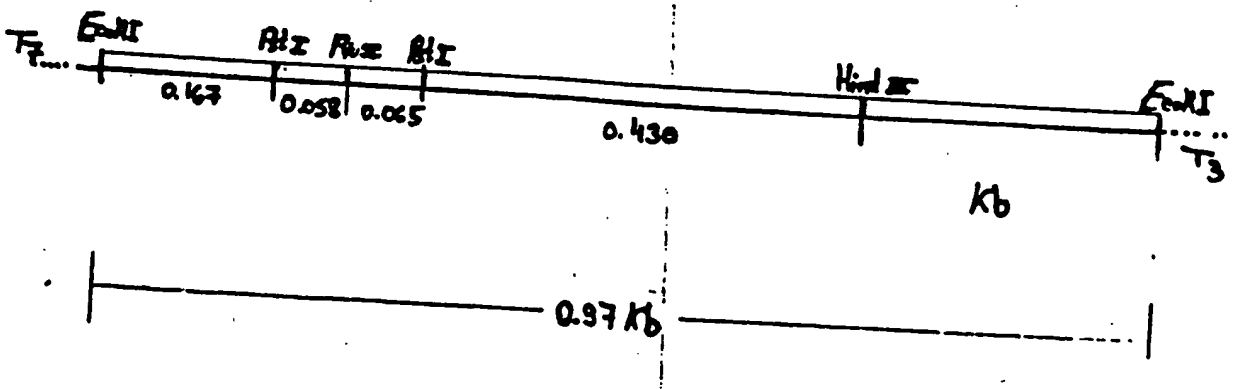

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Figure 2

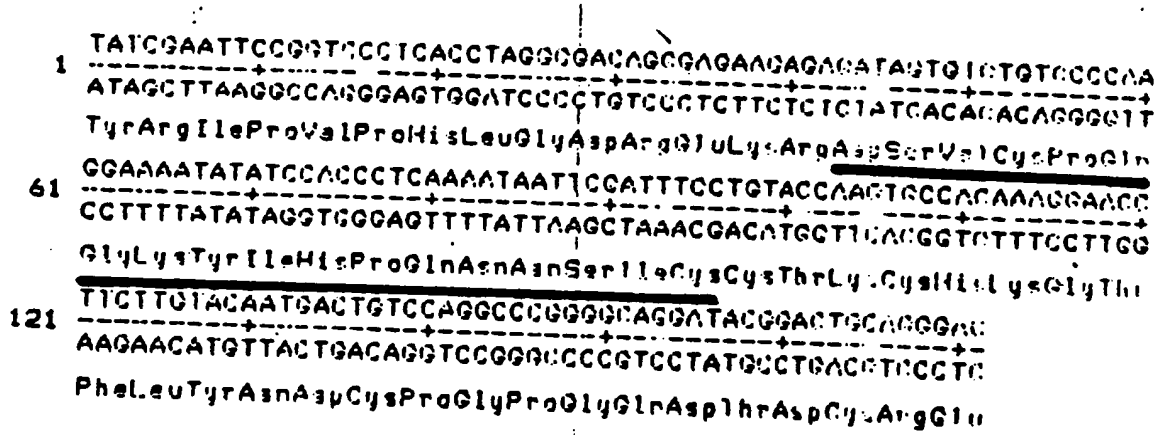

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Figure 3

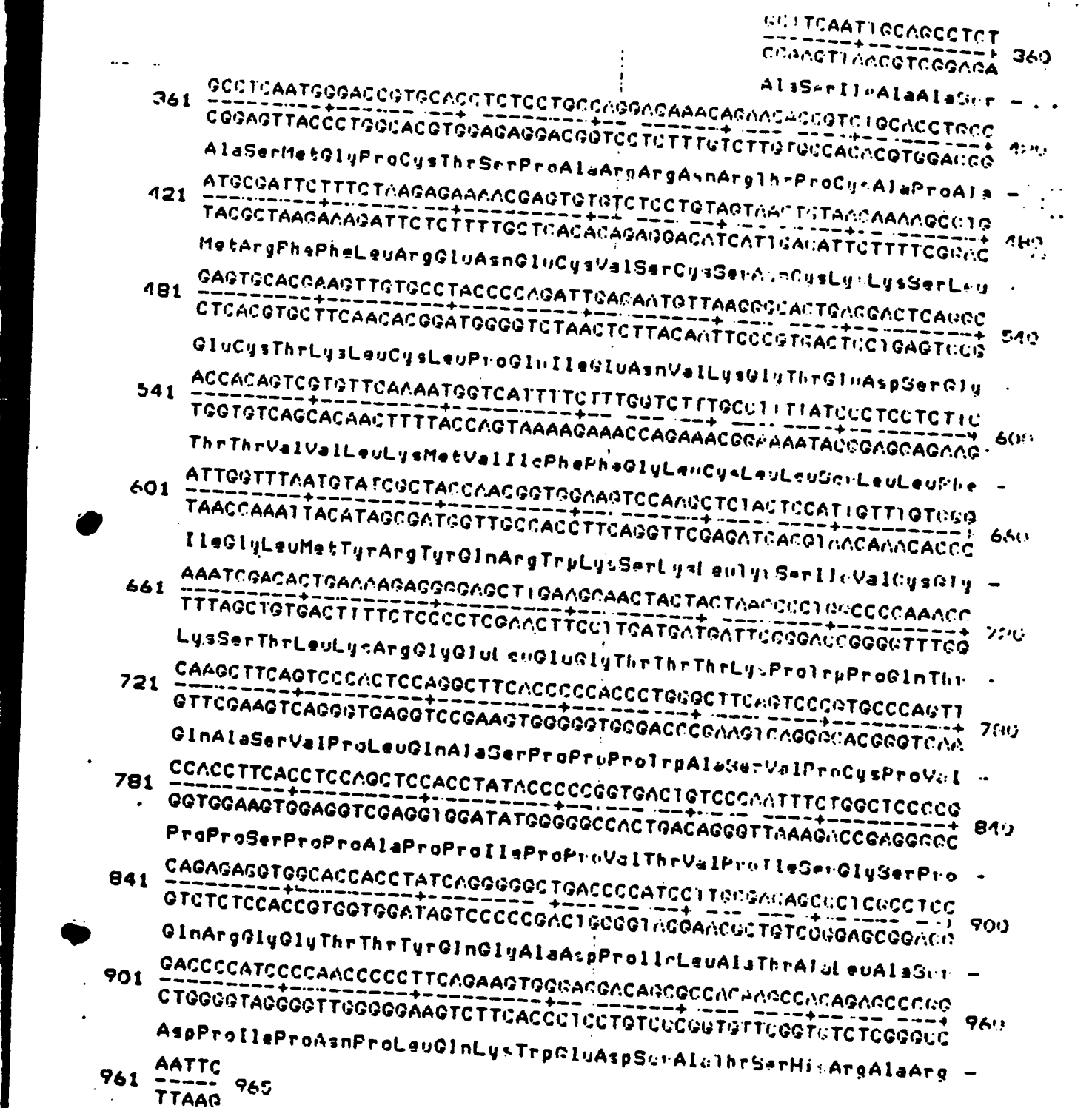

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FIGURE 4

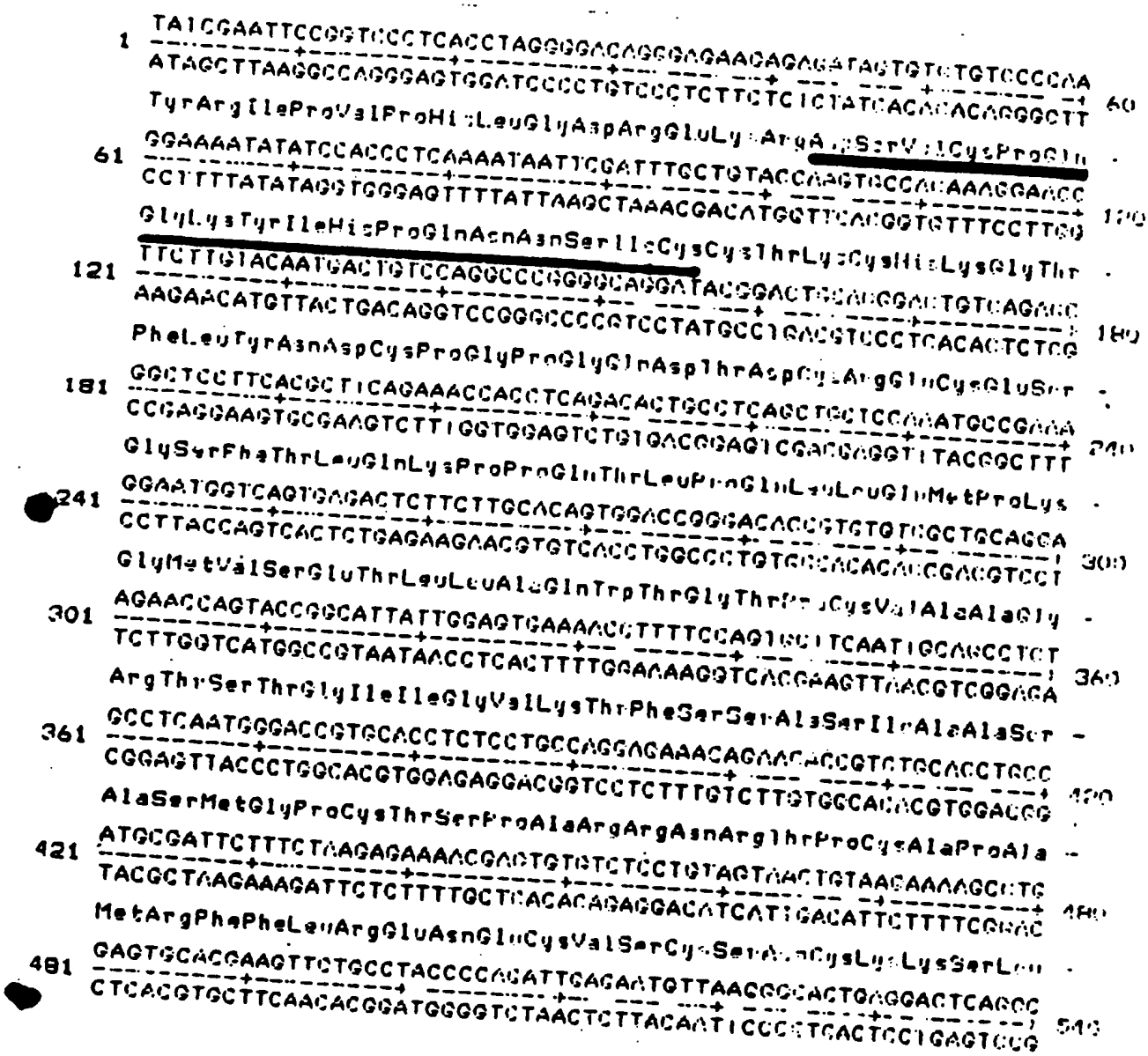

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FIGURE 4 (contd.)

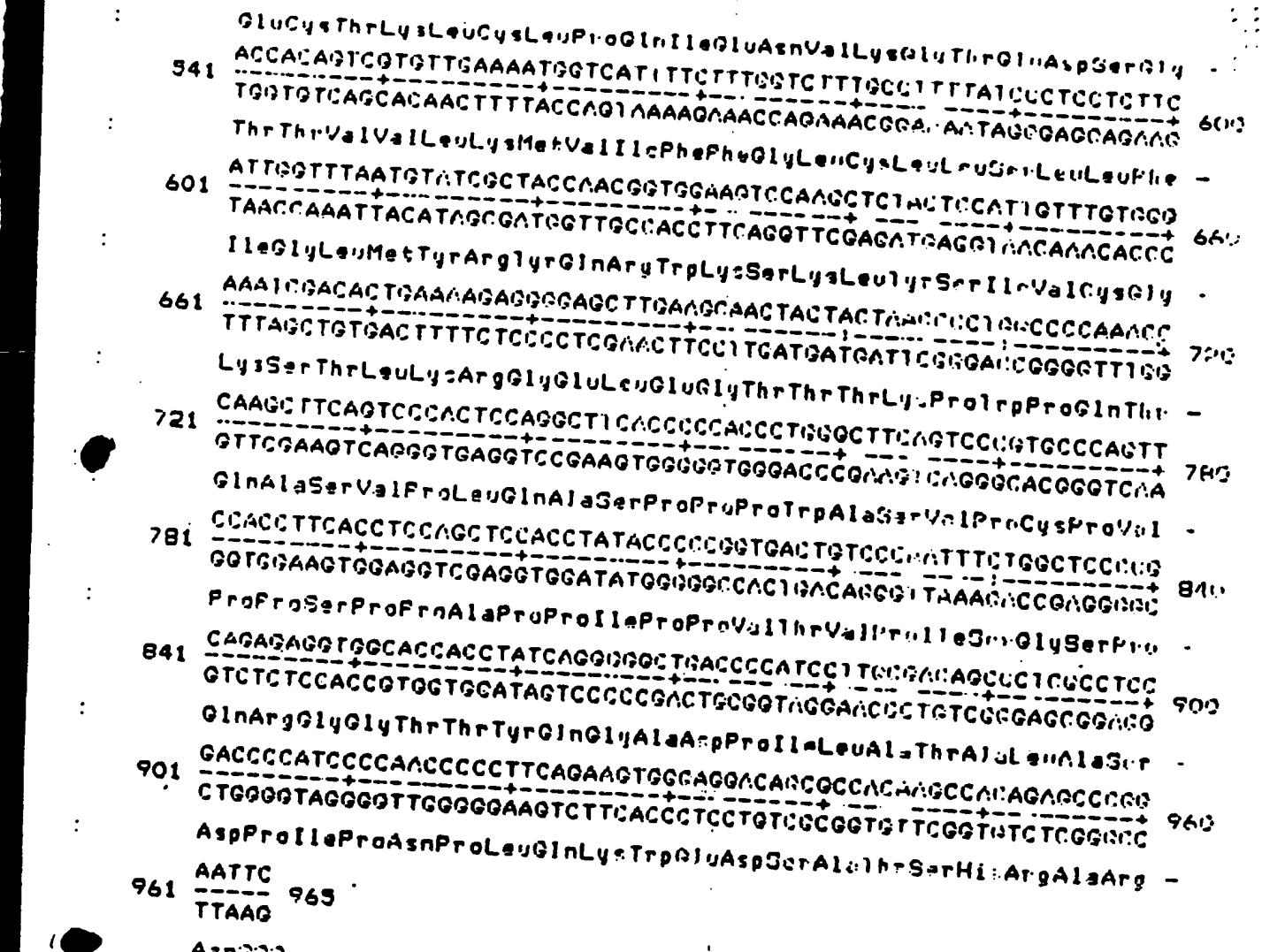

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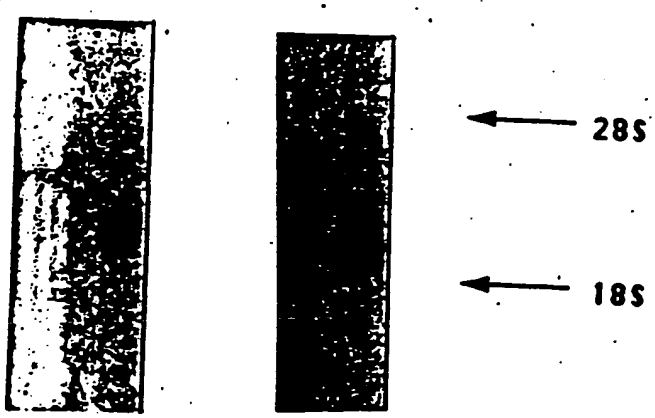
FIGURE 5

Detection of TBP1 mRNA
by northern blot hybridization



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Claims

1. A DNA molecule comprising a recombinant DNA molecule or a cDNA molecule coding for a protein comprising the amino acid sequence of TBP-I or a protein substantially homologous therewith.

2. A cDNA molecule according to claim 1 comprising a 1.08 Kb C2 insert of about 0.965 Kb, said insert providing a partial restriction map substantially as shown in Figure 1 upon digestion with restriction enzymes.

3. A cDNA molecule according to claim 1 or 2 comprising the partial nucleotide sequence shown in Figure 2 or a nucleotide sequence substantially homologous therewith.

4. A cDNA molecule according to claim 1 or 2 comprising the partial nucleotide sequence shown in Figure 3 or a nucleotide sequence substantially homogolous therewith.

5. A cDNA molecule according to claim 1 or 2 comprising the partial nucleotide sequences shown in Figures 2 and 3 or nucleotide sequences substantially homogolous therewith.

6. A cDNA molecule according to any of the preceding claims comprising the nucleotide sequence shown in Figure 4 or a nucleotide sequence substantially homologous therewith.

7. A cDNA molecule according to any of the preceding claims

encoding a protein comprising the amino acid sequence of TBP-I or a protein substantially homologous therewith.

8 A replicable plasmid vector comprising a DNA molecule according to any of the preceding claims.

9. A replicable plasmid vector according to claim 8 comprising the C2 insert of any of claims 2 to 8.

10. A bacterium transformed with a replicable plasmid vector according to claim 8 or 9.

11. A bacterium according to claim 10 which is an E.coli strain.

12. E.coli TG1 C2 having the deposit number CNCM I-917.

13. Oligonucleotide probes useful for picking up genes from cDNA libraries which code for proteins comprising the amino acid sequence of TBP-I, said probes having the formula:

1008 GGI GTC CCI TTC ATA TAA GTA GGI GT
T T G G G T

1009 GGA GTC CCA TTC ATA TA
C T C T G
T G
G T

1010 TTC ATA TAA GTA GGA GT
T G G G C
T G
T

14. DNA molecules hybridizable to all three oligonucleotide probes according to claim 13 and which code for a protein comprising

the amino acid sequence of TBP-I or a protein substantially homologous therewith.

15. mRNA isolated from U937 and HT29 cells having a size of about 2.5 Kb and being hybridizable with the C2 insert of claims 2 to 7.

16. The mRNA of claim 15 coding for a protein comprising the amino acid sequence of TBP-I or a protein substantially homologous therewith.

For the Applicants:

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Patent Attorney

Appendix E

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Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor

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Communicated by M.Revel

Two proteins which specifically bind tumor necrosis factor (TNF) have recently been isolated from human urine in our laboratory. The two proteins cross-react immunologically with two species of cell surface TNF receptors (TNF-R). Antibodies against one of the two TNF binding proteins (TBPI) were found to have effects characteristic of TNF, including stimulating phosphorylation of specific cellular proteins. Oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBPI were used to clone the cDNA for the structurally related cell surface type I TNF-R. It is notable that although this receptor can signal the phosphorylation of cellular proteins, it appears from its amino acid sequence to be devoid of intrinsic protein kinase activity. The extracellular domain of the receptor is composed of four internal cysteine-rich repeats, homologous to structures repeated four times in the extracellular domains of the nerve growth factor receptor and the B lymphocyte surface antigen CDw40. The amino acid composition and size of the extracellular domain of the type I TNF-R closely resemble those of TBPI. The COOH-terminal amino acid sequence of the four cysteine rich repeats within the extracellular domain of the type I TNF-R matches the COOH-terminal sequence of TBPI. Amino acid sequences in the extracellular domain also fully match other sequences found in TBPI. On the other hand, amino acid sequences in the soluble form of the type II TNF-R (TBPII), while indicating a marked homology of structure, did not suggest any identity between this protein and the extracellular domain of the type I TNF-R. CHO cells transfected with type I TNF-R cDNA produced both cell surface and soluble forms of the receptor. The receptor produced by CHO cells was recognized by several monoclonal antibodies against TBPI, reacting with several distinct epitopes in this molecule. These data suggest that the soluble forms of the TNF-Rs are structurally identical to the extracellular cytokine binding domains of these receptors and are consistent with the notion that the soluble forms are, at least partly, derived from the same transcripts that encode the cell surface receptors.

Key words: CDw40 antigen/cytokines/nerve growth factor/receptors/tumor necrosis factor

Introduction

Tumor necrosis factors TNF-α and TNF-β (lymphotoxin) are structurally related polypeptide cytokines, produced primarily by mononuclear leukocytes, whose effects on cell function constitute a major factor in the elicitation of the inflammatory response. The TNFs affect cells in different ways, some of which resemble the functional modes of other inflammatory mediators, like interleukin 1 (IL-1) and interleukin 6 (IL-6). What appears most distinctive regarding the activity of the TNFs is that many of their effects can result in cell and tissue destruction. Increasing evidence that over-induction of these destructive activities contributes to the pathogenesis of a number of diseases makes it of particular interest to elucidate their mechanisms and the ways they are regulated (Beutler and Cerami, 1988; Old, 1988). High affinity receptors, to which both TNF-α and TNF-β bind (Baglioni *et al.*, 1985; Beutler *et al.*, 1985; Kull *et al.*, 1985; Tsujimoto *et al.*, 1985; Aggarwal *et al.*, 1986; Israel *et al.*, 1986) play a key role in the initiation and control of the cellular response to these cytokines. These receptors are expressed on the surfaces of a variety of different cells. Studies showing that antibodies reacting with their extracellular portions affect cells in a manner very similar to the TNFs demonstrate that the receptors and cellular components associated with them are sufficient to provide the intracellular signalling for the effects of the TNFs (Engelmann *et al.*, 1990a; Espevik *et al.*, 1990). Other studies have shown that molecules related to the TNF receptors (TNF-Rs) also exist in soluble forms. Two immunologically distinct species of such soluble TNF-Rs, TBPI and TBPII, were recently isolated from human urine (Engelmann *et al.*, 1989, 1990b; Olsson *et al.*, 1989; Seckinger *et al.*, 1989a). Immunological evidence indicated that the two proteins are structurally related to two molecular species of the cell surface TNF-R (the type I and type II receptors, respectively). Antibodies to each of the two soluble proteins were shown to block specifically the binding of TNF to one of the two receptors and could be used to immunoprecipitate the receptors. Antibodies against one of the two soluble proteins (TBPI) were also found to induce effects characteristic of TNF in cells which express the immunologically cross-reactive cell receptors (Engelmann *et al.*, 1990a, 1990b). Like the cell surface receptors for TNF, the soluble forms of these receptors specifically bind TNF and can thus interfere with its binding to cells. It was suggested that they function as physiological inhibitors of TNF activity (Engelmann *et al.*, 1989; Olsson *et al.*, 1989; Seckinger *et al.*, 1989a).

In the present study we explored the structural relationship of the soluble and cell surface forms of the TNF-Rs further by determining amino acid sequences of the soluble forms and by using amino acid sequence data for one of the soluble receptors to clone the cDNA which encodes this protein. Initial information on the mechanism of formation

of the soluble receptors was gained by examining the expression of this cDNA in transfected CHO cells.

Results

Cloning of the cDNA for the type I TNF-R

To clone the cDNAs which code for the TNF-binding protein, TBPI, and its related TNF receptor, we screened several cDNA libraries, using three overlapping oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBPI (Figure 1A). In a λGT11 library derived from the mRNA of human colon (randomly primed, Clontech, Palo Alto, CA), we detected four recombinant phages which hybridized with the three probes. The inserts in these four phages were similar in size, and were found to overlap by restriction mapping and sequence analysis. Complete analysis of the sequence of the longest of the four (C2 in Figure 1B) revealed an open reading frame extending over its entire length. A polypeptide chain encoded in this reading frame fully matches the NH_2-terminal amino acid sequence of TBPI. Neither an initiation nor a stop codon was found in the C2 insert. Rescreening the colon cDNA library using another probe corresponding to a sequence found in C2 (see Materials and methods) yielded several other recombinant phages containing inserts that overlap with the C2 insert. However, none of them provided further sequence information on the cDNA in the 5′ or the 3′ directions. In a λZAP cDNA library derived from the mRNA of CEM lymphocytes (Foley *et al.*, 1965) [oligo (dT) and randomly primed, Clontech] five phages hybridizing with this probe were detected, which contained significantly longer inserts than C2. The longest insert (E13, Figure 1B) was sequenced in its entirety (Figure 1D) and was found to contain the C2 sequence (nucleotides 346–1277 in Figure 1D) within one long open reading frame of 1365 bp, flanked by untranslated regions of 255 and 556 nucleotides at its 5′ and 3′ ends respectively. The potential ATG initiation site, occurring at positions 256–258 in the nucleotide sequence, (denoted by an asterisk in Figure 1d) is preceded by an upstream in-frame termination codon at bases 244–246. The start location is in conformity with one of the possible alternatives for the translation initiation consensus sequence (GGCATGG, nucleotides 253–259; Kozak, 1987).

There is no characteristic poly(A) addition signal near the 3′ end of the cDNA. The sequence ACTAAA, at nucleotides 2045–2050, may serve as an alternative to this signal, but with low efficiency (Sheets and Wickens, 1989). At nucleotides 1965–2000, there are six consecutive repeats of the sequence G(T)n (n varying between 4 and 8). Similar sequences have also been observed in the 3′ noncoding regions of the cDNAs of some members of the *jun* family, which are also devoid of the characteristic poly(A) signal (Ryder *et al.*, 1988, 1989). The 3′ end has a 15 base poly(A) tail.

The size of the protein encoded by the cDNA (~50 kd) is significantly larger than that of TBPI (Engelmann *et al.*, 1989; Olsson *et al.*, 1989; Seckinger *et al.*, 1989a). A hydropathy index computation (Kyte and Doolittle, 1982) of the deduced amino acid sequence of the protein (Figure 1C) revealed two major hydrophobic regions (see round-ended boxes in Figure 1D). One, at its NH_2-terminus, is apparently the signal peptide whose most likely cleavage site

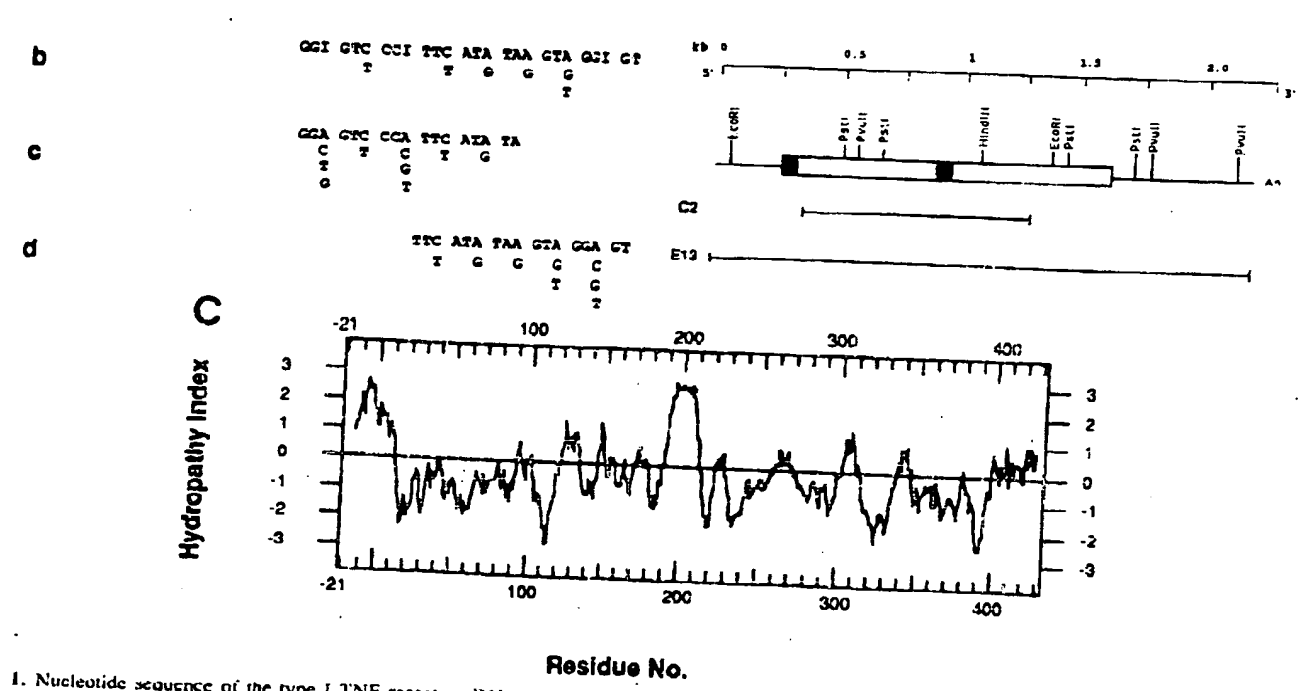

Fig. 1. Nucleotide sequence of the type I TNF receptor cDNA and the predicted amino acid sequence of the encoded protein. (A) The probes used for screening for the cDNA: (a) The NH_2-terminal amino acid sequence of TBPI. (b) Synthetic oligonucleotide probes, designed on the basis of the NH_2-terminal amino acid sequence, used for initial screening. (c) and (d) Probes overlapping with the probes presented in b, used to confirm the validity of clones isolated in the initial screening. (B) Schematic presentation of the cDNA clones isolated from a human colon (C2) and from CEM-lymphocyte (E13) libraries and a diagram of the complete cDNA structure. Untranslated sequences are represented by a line. Coding regions are boxed. The shaded portions represent the sequences which encode the signal peptide and the transmembrane domains. (C) Hydropathy profile of the predicted amino acid sequence of the TNF receptor. Hydrophobicity (above the line) and hydrophilicity (below the line) values were determined using the sequence analysis software package of the University of Wisconsin genetic computer group (UWGCG) according to Kyte and Doolittle

D

1 CGGCCCAGTGATCTTGA 17

18 ACCCCAAAGCCCACAACTGGAGCCTCAGTCCAGAGAATTCTGAGAAAATTAAAGCAGAGAGGAGGGGAGAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAACCCTCAA 136

137 CTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCCCCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGGGGGAGTGAGAGGCCATAGCTGTCTGGC 255

-21 -10 -1 +1

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu

256 ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG 345

*

10 20 30

Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr

346 GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC 435

40 50 60

Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr

436 AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC 525

70 80 90

Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp

526 GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC 615

100 110 120

Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu

616 CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC 705

130 140 150

Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val

706 AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC 795

160 170 180

Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr

796 TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC 885

190 200 210

Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys

886 ACA GTG CTG TTG CCC CTG GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG 975

220 230 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn

976 TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT AAG CCC CTG GCC CCA AAC 1065

250 260 270

Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr

1066 CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC 1155

280 290 300

Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala

1156 CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC 1245

310 320 330

Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp Thr Asp Asp Pro Ala Thr Leu Tyr

1246 TCC GAC CCC ATC CCC AAC CCC CTT CAG AAG TGG GAG GAC AGC GCC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAC 1335

340 350 360

Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu Ile Asp Arg Leu Glu Leu

1336 GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG ATC GAT CGG CTG GAG CTG 1425

370 380 390

Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala Thr Leu Glu Leu

1426 CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG CGG CGC ACG CCG CGG CGC GAG GCC ACG CTG GAG CTG 1515

400 410 420

Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro Pro Ala

1516 CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GCC GCC CTC CCG CCC GCG 1605

430

Pro Ser Leu Leu Arg End

1606 CCC AGT CTT CTC AGA TGA GGCTGCGCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTCTGGAAAGGAGGGGTCCTGCAGGGGCAAGC 1718

*

1719 AGGAGCTAGCAGCCGCCTACTTGGTGCTAACCCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCCGACAGTCAGCGCTGTGCGCGCGGAGAGAGGTGCGCCGTGGGCTCAA 1837

1838 GAGCCTGAGTGGGTGGTTTGCGAGGATGAGGGACGCTATGCCTCATGCCCGTTTTGGGTGTCCTCACCAGCAAGGCTGCTCGGGGGCCCCTGGTTCGTCCCTGAGCCTTTTTCACAGTG 1956

1957 CATAAGCAGTTTTTTTTGTTTTTGTTTTGTTTTGTTTTGTTTTTAAATCAATCATGTTACACTAATAGAAACTTGGCACTCCTGTGCCCTCTGCCTGGACAAGCACATAGCAAGCTGAA 2075

2076 CTGTCCTAAGGCAGGGGCGAGCACGGAACAATGGGGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAAAAAAAAAA 2176

(1982). The curve is the average of the hydrophobicity index for each residue over a window of nine residues. **(D)** Nucleotide and predicted amino acid sequences of the TNF receptor. The presumptive start and stop signals are denoted by asterisks; the three sequences derived from TBPI by broken overlining; the transmembrane and leader domains by round-ended boxes; and the four repetitive sequences in the extracellular domain by thick underlining. Cysteine residues are boxed. Glycosylation sites are overlined and the presumptive polyadenylation signal is underlined.

Table I. Amino acid sequences of TBPI and TBPII

TBP I:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CNBr-1 (=N-terminus)	NH_2	Asp	Ser	Val	Cys	Pro	Gln	Gly	Lys	Tyr	Ile	His	Pro	Gln	---
CNBr-2	NH_2	Gly	Gln	Val	Glu	Ile	Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	---
C-terminus	---	Ile	Glu	Asn	COOH										

TBP II:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
N-terminus	NH_2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg	Leu	Arg	Glu	Tyr	Tyr	---
TRP 35	NH_2	Leu	Cys	Ala	Pro	Leu	Arg	Lys																
TRP 39/1	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg														
TRP 39/2	NH_2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	—											
TRP 44/1	NH_2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	Ser	---										
TRP 44/2	NH_2	Ser	Cys	Gly	Pro	Ser	Tyr	Pro	Asp	—														
TRP 46/1	NH_2	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg										
TRP 46/2	NH_2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---									
TRP 50	NH_2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---									
TRP 53/1	NH_2	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg								
TRP 53/2	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg														
TRP 54/1	NH_2	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	Lys														
TRP 54/2	NH_2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg						
TRP 60	NH_2	Ile	Cys	Thr	Cys	Arg	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	---									
TRP 62	NH_2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	---			
TRP 65	NH_2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	---			
TRP 67	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys			
TRP 84	NH_2	Thr	Ser	Asp	Thr	Val	Cys	Asp	Ser	Cys	Glu	Asp	Ser	Thr	Tyr	Thr	Gln	Leu	Trp	---				

(Von Heijne, 1986) lies between the glycine and isoleucine residues designated in Figure 1D as −1 and +1 respectively. The other major hydrophobic domain, located between residues 191 and 213, is flanked at both ends by several charged amino acids, characteristic of a membrane anchoring domain (Pidgeon *et al.*, 1989). As in several other transmembrane proteins, the amino acids confining the hydrophobic domain at its COOH-terminal are basic. The transmembrane domain bisects the predicted protein into almost equally sized extracellular and intracellular domains.

The extracellular domain has three putative sites for asparagine-linked glycosylation (overlined in Figure 1D). Assuming that the amount of oligosaccharides in the extracellular domain is similar to that reported in TBPI (Seckinger *et al.*, 1989b), the molecular size of the mature protein is very similar to that estimated for the type I receptor (~58 kd) (Hohmann *et al.*, 1989; Engelmann *et al.*, 1990a).

Features of the predicted amino acid sequence in the type I TNF-R and relationship to the structure of TBPI and TBPII

The amino acid sequence of the extracellular domain of the protein encoded by the E13 cDNA fully matches several determined TBPI amino acid sequences (Table I). It contains the NH_2-terminal amino acid sequence of TBPI at amino acids 20−32 (compare Figure 1D and 1A a), a sequence corresponding to the COOH-terminus of TBPI at amino acids 178−180, and also, adjacent to the first methionine located further downstream in the encoded protein, a sequence identical to the NH_2-terminal amino acid sequence of a cyanogen-bromide cleavage fragment of TBPI (broken lines in Figure 1D). There is also a marked similarity in amino acid composition between the extracellular domains of the receptor and TBPI (Table II). The most salient feature of this amino acid composition is a very high content of cysteine residues (shown boxed in Figure 1D). The positioning of the cysteine residues as well as of other amino acids within the extracellular domain displays a four-fold repetition pattern (Figure 2 and underlined in Figure 1D). As shown in Figure 2, there is a marked homology between this four-

Table II. Similarity of the amino acid compositions of the TNF binding protein TBPI and a corresponding region in the extracellular domain of the TNF-R (type I)

Amino acid	mol/100 mol of amino acids	
	TBPI[a]	Residues 20−180 in the extracellular domain[b]
Ala	1.7	1.2
Cys	12.8	14.9
Asp + Asn	10.9	11.1
Glu + Gln	13.9	12.4
Phe	3.2	3.1
Gly	6.3	5.6
His	4.4	4.3
Ile	2.8	2.5
Lys	6.2	6.2
Leu	8.0	6.8
Met	0.4	0.6
Pro	3.8	3.1
Arg	4.7	4.3
Ser	8.1	9.3
Thr	6.1	6.2
Val	4.2	4.3
Trp	—	0.6
Tyr	2.4	3.1

[a]According to Olsson *et al.*, 1989.
[b]Residue 20 corresponds to the NH_2-terminal amino acid of TBPI. Residue 180 is the COOH-terminal residue of TBPI.

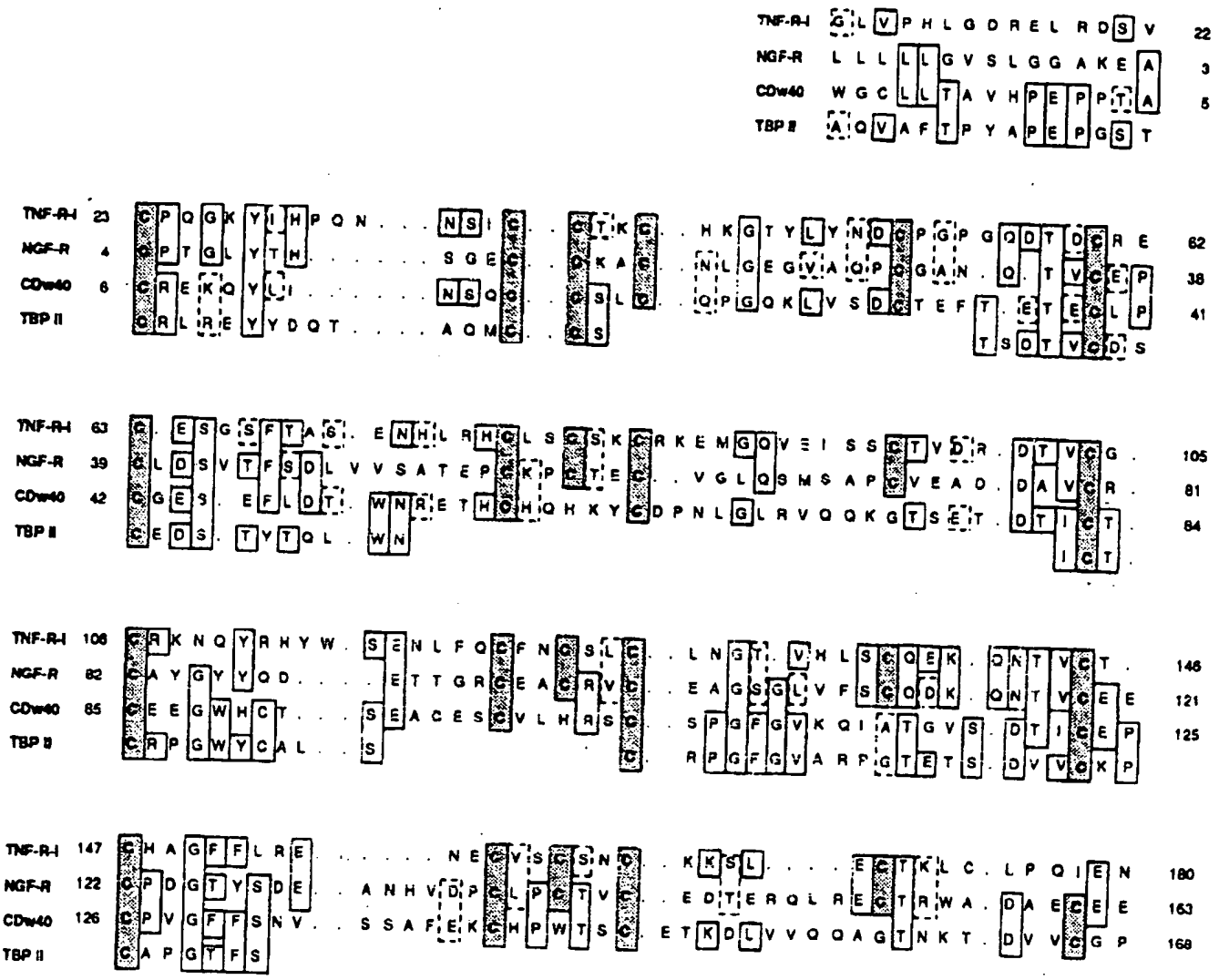

Fig. 2. Internal cysteine-rich repeats in the extracellular domain of the TNF-R and their alignment with the homologous repeats in the extracellular domains of human NGF-R and the CDw40 antigen and with sequences of amino acids present in TBPII. The amino acid sequences (one-letter symbols) are aligned for maximal homology. Dashes indicate gaps introduced to optimize the alignment. Identities in sequences are shown in boxes. Conservative substitutions (I = L = V; D = E; K = R = H; T = S; G = A; N = Q) are boxed with dotted lines. The positions of the amino acids within the receptors are denoted in the right and left hand margins. The sequences in TBPII (Table I and Materials and methods) were optimally aligned with the sequences of the other three proteins presented in this figure, using the 'Best Fit' alignment program in the UWGCG software package.

domain structure and sequences found in the extracellular domain of the receptor for the nerve growth factor (NGF-R) (Johnson *et al.*, 1986; Radeke *et al.*, 1987) and also, to a somewhat lower degree, to sequences in the extracellular domain of the recently cloned receptor-like B cell antigen CDw40 (Braesch-Andersen *et al.*, 1989; Stamenkovic *et al.*, 1989). The amino acid sequence within the extracellular domain of the TNF-R, which corresponds to the COOH-terminal sequence of TBPI (see Table I and Figure 7), is located at the COOH-terminus of the cysteine-rich repeat region. The sequence corresponding to the NH_2-terminal sequence of TBPI corresponds to a sequence located a few amino acids upstream of the NH_2-terminal end of this region (broken lines in Figure 1D) in the extracellular domain.

In contrast to the identity of amino acid sequences between TBPI and the extracellular domain of the type I receptor, sequences examined in the soluble form of the type II TNF-R (TBPII, Table I) were not identical to any sequence in the type I TNF-R. This finding is expected, considering the lack of immunological cross-reactivity between the two receptors (Engelmann *et al.*, 1990b). However, as demonstrated in Figure 2, the sequences in TBPII have a significant homology of structure with the four-fold cysteine-rich repeat region in the extracellular domains of the type I TNF-R, the NGF-R and the CDw40 protein. The similarity between TBPII and the CDw40 protein is particularly notable and seems to extend also to a region within the two proteins which protrudes from the cysteine-rich structures in the NH_2 direction (Figure 2).

In contrast to the very high content of cysteine residues in the putative extracellular domain of the type I TNF-R, there are only five cysteine residues in the intracellular domain. Between the two which are proximal to the transmembrane domain (positions 227 and 283) extends a stretch of 55 amino acids which is rich in proline residues (16% of the residues) and even richer in serine and threonine residues (36%), most located very close to or adjacent to each other. The consensus sequence Gly-x-Gly-x-x-Gly

nucleotide binding proteins (Kamps *et al.*, 1984) is not present in the intracellular domain.

Expression of the type I TNF-R cDNA

To explore the relation between the protein encoded by the E13 cDNA and TBPI further, we expressed this protein in CHO cells. The E13 cDNA was introduced into an expression vector and was cotransfected with a recombinant vector containing the dihydrofolate reductase (DHFR) cDNA into DHFR-deficient cells. After selection by growth in a nucleotide-free medium, individual clones were amplified by growth in the presence of methotrexate. A number of clones which react with several monoclonal antibodies that bind to spatially distinct epitopes in TBPI were detected (Figure 3). Expression of the protein was correlated with an increase in specific binding of human TNF to the cells (Table III).

Applying a sensitive immunoassay for TBPI, in which polyclonal antibodies and a monoclonal antibody against this protein were employed, we could also detect a soluble form of the protein in the growth medium of CHO cells, which express the human TNF-R on their surface (Table III). All of five different CHO clones which expressed the TNF-R produced this soluble protein. Several other transfected clones which did not express the cell surface receptor did not produce its soluble form either (not shown). When analyzed by reversed phase HPLC, the CHO-produced soluble TNF-R eluted as a single peak, with a retention time identical to that of TBPI (Figure 4).

Northern blot analysis using the E13 cDNA as a probe

To gain information on the transcripts which encode the type I TNF-R, we tested mRNAs from cells of differing origin for their ability to hybridize with the E13 cDNA. As shown in Figure 5, in all the cell types, including the HT29 cells, which continuously secrete a soluble form of the type I TNF-R (Aderka,D., Nophar,Y., Engelmann,H. and Wallach,D., manuscript in preparation), only a single hybridizing transcript was detectable, in all cases of the same size i.e. ~2300 bp, corresponding to the full length of the cDNA. Interestingly, significant amounts of this type

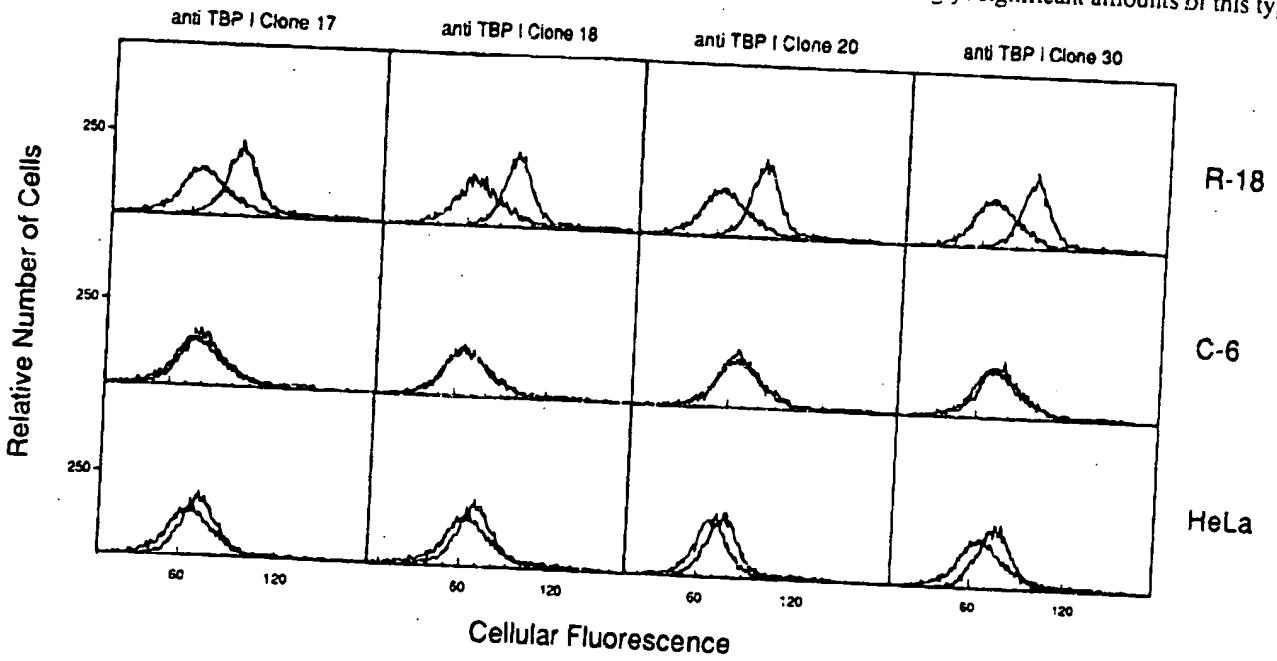

Fig. 3. Detection of type I TNF-R using monoclonal antibodies to TBPI in CHO cells transfected with E13 cDNA. CHO cells, clones R-18 (transfected with an expression vector in which the E13 cDNA was placed under the control of an SV40 promotor) and C-6 (control; a clone of cells transfected with an expression vector in which E13 was placed in the inverse orientation), and HeLa cells were stained with the anti-TBPI monoclonal antibodies 17, 18, 20 and 30 followed by incubation with FITC conjugated anti-mouse F(ab). Fluorescence intensity is compared with that observed when a mouse monoclonal antibody against TNF was used in the first step of the staining as a control.

Table III. Expression of the cell surface and soluble forms of human type I TNF-R in CHO cells

CHO cell clone	Specific binding of TNF (c.p.m./10^6 cells)	Cells expressing human cell surface TNF-R (% fluorescent cells)	Human soluble type I TNF receptors (pg/ml)
nontransfected	180 ± 45	<1%	<0.03
C6	175 ± 50	<1%	<0.03
R-16	550 ± 60	73%	30
R-18	610 ± 40	89%	49

The R-16 and R-18 clones consist of cells transfected with a recombinant expression vector containing E13 cDNA. C-6 cells were transfected with a control vector (see Figure 3). Binding of radiolabeled TNF to the cells was determined in quintuplicate samples. Detection of immunoreactive receptors on the surface of the cells was carried out using combined 17, 18 and 20 anti-TBPI monoclonal antibodies. Results are expressed as percentage of fluorescent cells (background values, obtained by staining the cells with an anti-TNF monoclonal antibody, are subtracted).

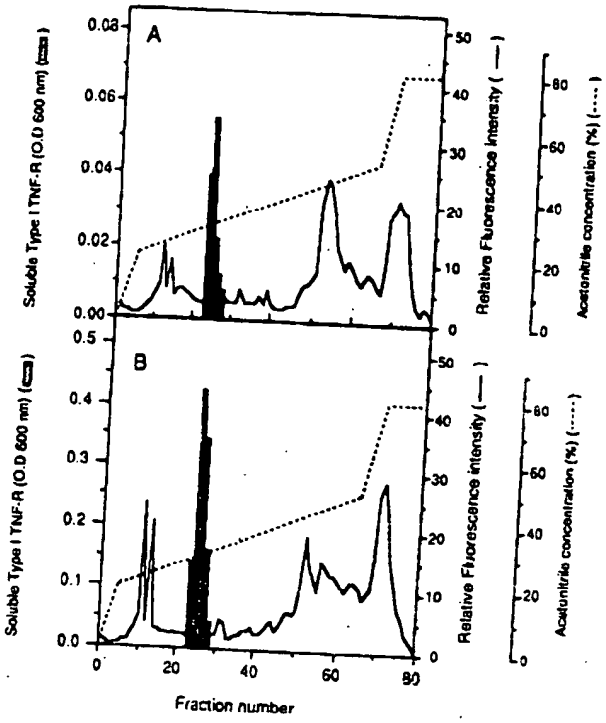

Fig. 4. Reversed phase HPLC of the CHO-produced, soluble form of the type I TNF-R. A concentrate of the conditioned medium of the CHO R-18 clones (see Figure 3) and a concentrate of the CHO C-6 clone to which 3 ng pure TBPI was added were applied to an Aquapore RP300 column. Elution was performed with a gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid (---). Fractions were examined for content of protein (——) and of the soluble form of the type I by ELISA (▩), as described in Materials and methods. None of the eluted fractions of a concentrate of the CHO C-6 clone without addition of TBPI was found to contain any detectable amounts of the soluble form of the receptor (not shown).

Fig. 5. Detection of the mRNA for the type I TNF-R by Northern blotting analysis. Hybridization of total RNA (25 μg/lane) from cells of the Jurkat (Gillis and Watson, 1980), CEM (Foley *et al.*, 1965), KG-1 (Koeffer and Golde, 1978), Daudi (Klein and Klein, 1968), K-562 (Lozzio and Lozzio, 1975), U-937 (Sundstroem and Nilsson, 1976), A-673 (Giard *et al.*, 1973), Hep 3B (Aden *et al.*, 1979), HT-29 (Fogh and Trempe, 1975), and HeLa (Gey *et al.*, 1952) lines with the ^{32}P-labeled E13 insert was carried out as described in Materials and methods. 28S and 18S refer to ribosomal RNA size markers.

I TNF-R mRNA could also be detected in the U937 cells, in which the prevalent TNF-R is type II (Engelmann *et al.*, 1990b), suggesting that post-transcriptional mechanisms take part in the control of the expression of the type I receptor.

Evidence for the involvement of type I TNF-R in stimulation of protein phosphorylation by TNF

Treating cells with TNF results in a rapid increase in the phosphorylation of certain specific cellular proteins [illegible]

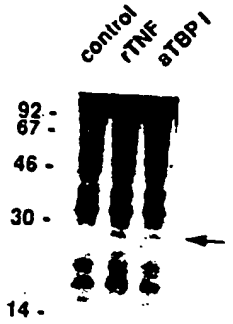

Fig. 6. Involvement of the type I TNF-R in stimulation of protein phosphorylation in cells. Effects of TNF-α (1000 U/ml) and rabbit antiserum to TBPI (1:1000) on the phosphorylation of proteins with a mol. wt of 27 kd in HeLa cells. Untreated cells served as a control. The 27 kd protein(s) are indicated with an arrow on the right and the migration of molecular weight markers (Amersham, UK) is shown on the left. Normal rabbit serum had no effect at a dilution of 1:1000 (not shown).

some with a mol. wt of ~27 kd (Hepburn *et al.*, 1988; Kaur and Saklatvala, 1988; Schutze *et al.*, 1989). Since it is apparent from the sequence data of the intracellular domain of the type I TNF-R that this receptor is devoid of intrinsic protein kinase activity, it was of interest to examine the extent to which this receptor is involved in TNF-mediated protein phosphorylation events. Antibodies to TBPI induce various effects in cells which are characteristic of TNF. This activity was shown to be correlated with the ability of the antibodies to cross-link the type I TNF-R molecules (Engelmann *et al.*, 1990a). As shown in Figure 6, treating HeLa cells which express the type I TNF-R (Engelmann *et al.*, 1990b) with antibodies to TBPI induced, as does TNF, a marked increase in the phosphorylation of protein(s) with a mol. wt of 27 kd, confirming that the type I TNF-R is involved in this effect.

Discussion

There is accumulating evidence for the natural occurrence of soluble forms of cell surface receptors. Such forms have been identified, for example, for the receptors to interleukin-2 (IL-2) (Rubin *et al.*, 1985; Osawa *et al.*, 1986), growth hormone (Leung *et al.*, 1987), NGF (DiStefano and Johnson, 1988), interleukin-6 (Novick *et al.*, 1989), interferon-γ (Novick *et al.*, 1989) and tumor necrosis factor (Engelmann *et al.*, 1989, 1990b; Olsson *et al.*, 1989; Seckinger *et al.*, 1989a). Yet knowledge of the exact structure of these soluble receptors and of the mechanisms of their formation is still limited. The most thoroughly characterized so far is the soluble form of the 55 kd receptor for IL-2. Based on detailed sequence analysis and studies of its mode of formation in cultured cells, it was suggested that it is derived from the cell surface form of the receptor by proteolytic cleavage (Robb and Kutny, 1987).

A different mechanism for the formation of soluble receptors was proposed in two recent studies describing the cloning of the cDNAs for the receptors to IL-4 and IL-7. Besides cDNA clones encoding the full length receptors, clones which encode truncated, soluble forms of these receptors were also isolated in these studies. It was suggested that these latter clones are derived [illegible]

transcribed from the same genes which encode the cell surface forms, but differently spliced (Mosley *et al.*, 1989; Goodwin *et al.*, 1990).

Data presented in our study are consistent with the notion that TBPI—the soluble form for the type I TNF-R—constitutes a fragment of the cell surface form of this receptor corresponding to its extracellular domain. The receptor is recognized by several monoclonal antibodies to TBPI which interact with several spatially distinct epitopes in this protein (the present study and Engelmann *et al.*, 1990a). The amino acid sequence in the extracellular domain fully matches several sequences present in TBPI. Furthermore, the amino acid composition of the region within the extracellular domain which extends between those residues which correspond to the NH_2- and COOH-termini of TBPI is very similar to the amino acid composition reported for TBPI. There is also a similarity in size between TBPI and this part of the receptor [taking into account that about a third of the TBPI molecule consists of oligosaccharides (Seckinger *et al.*, 1989b)]. Particularly informative with regard to the mechanism of formation of TBPI is the finding that a soluble form of the type I TNF-R is produced by CHO cells transfected with the TNF-R cDNA. This finding implies that cells possess some mechanism(s) which allow(s) the formation of the soluble form of the TNF-R from the same transcript that encodes the cell surface form. There is no indication from the data of this study for the existence of transcripts which specifically encode soluble forms of TNF-R. Northern blot analysis did not reveal transcripts smaller than the full size of the TNF-R mRNA in any of the cells examined, not even in the HT29 cells, which continuously release significant amounts of a soluble form of the type I TNF-R into the culture medium. Furthermore, sequence and restriction mapping analyses of the various cDNA clones isolated in this study together with the C2 and E13 clones failed to reveal any difference in structure, besides differences in size, between these clones and the E13 cDNA (data not shown). The amino acid sequence data of TBPI also provide no indication of the existence of transcripts specific to this protein. Soluble receptors produced from alternatively spliced transcripts, as suggested for the IL-4 and IL-7 receptors, are expected to have unique COOH-terminal sequences (Mosley *et al.*, 1989; Goodwin *et al.*, 1990). The COOH-terminal sequence of TBPI was found to be identical to a sequence found in the cell surface receptor. Still, the existence of a minor population of transcripts which specifically code for soluble forms of TNF-Rs in amounts below the limit of detection of the techniques employed, although not supported by the data presented in this study, cannot be excluded.

The low rate of production of the soluble form of the type I TNF-R by the E13-transfected CHO cells does not necessarily reflect maximal activity. In HT29 cells, the spontaneous release of a soluble form of type I TNF-R occurs at about a 10-fold higher rate than that observed with the CHO-R-18 clone (data not shown). Furthermore, a recent study (Porteu and Nathan, 1990) indicates that the mechanism of formation of the soluble TNF-R can be effectively enhanced by certain specific stimuli. Stimulation of human neutrophils with N-formyl Met-Leu-Phe, or with several other physiological stimuli, was found to result, within a few minutes, in an extensive decrease of the cell-surface expressed TNF-R and an accompanying release of a soluble form of these receptors, similar in size to TBPI.

A likely mechanism whereby soluble forms of TNF receptors can be derived from the same transcripts which encode the cell surface forms is proteolytic cleavage. Indeed, flanking the amino acid residue which corresponds to the NH_2-terminus of TBPI there are, within the amino acid sequence of the receptor, two basic amino acid residues (Lys-Arg) which can serve as a site of cleavage by trypsin-like proteases. The identity of the proteases which might cause cleavage to take place at the COOH-terminus of TBPI is not known. In view of the marked structural homology between the extracellular domain of the type I TNF-R and the soluble form of the type II TNF-R (TBPII) as well as the homology with the extracellular domain of the NGF-R, for which existence of a soluble form has been also documented (DiStefano and Johnson, 1988; Zupan *et al.*, 1989), it is tempting to speculate that a common mechanism of cleavage and similar cleavage sites are involved in the formation of the soluble forms of those three receptors. Such a mechanism can have a dual effect on cell response to TNF. Its activation may result in the suppression of the response both in those cells in which it functions—as a consequence of the decrease in their intact cell surface receptors—as well as in other cells because of the ability of the released, soluble form of the receptor to sequestrate TNF. Detailed analysis of the biosynthesis of TNF-R, facilitated by use of cells transfected with TNF-R cDNA in vectors dictating its overexpression, should provide further information on the mechanism and functional implications of the formation of its soluble form.

Materials and methods

Determination of amino acid sequences within the TNF-binding proteins TBPI and TBPII

The TNF binding proteins TBPI and TBPII were isolated from concentrated preparations of urinary proteins, as described previously (Engelmann *et al.*, 1990b) by ligand (TNF) affinity chromatography followed by reversed phase HPLC. TBPI was cleaved with cyanogen bromide, yielding two peptides which, following reduction and alkylation (Andrews and Dixon, 1987), were isolated by reversed phase HPLC. The two peptides (CNBr-1 and CNBr-2 in Table I) were subjected to NH_2-terminal sequence analysis on a pulsed liquid gas phase protein microsequencer (Model 475A, Applied Biosystems Inc., Foster City, CA). The sequence found for one of the peptides (CNBr-1) was identical to the NH_2-terminal sequence of the intact TBPI protein (Engelmann *et al.*, 1989, 1990b).

The COOH-terminal amino acid sequence of TBPI was determined by digestion of the protein with carboxypeptidase Y followed by sequential analysis of the released amino acids. A sample of pure TBPI (32 μg) was mixed with 1 nmol norleucine, as an internal standard, dried thoroughly and resuspended in 8 μl 0.1 M sodium acetate buffer, pH 5.5, containing 0.8 μg carboxypeptidase Y (Sigma, St Louis, MO). Digestion was performed at room temperature. 2 μl aliquots withdrawn at various time points were acidified by adding 3 μl of 10% acetic acid to each, followed by addition of 15 μl 0.5% EDTA. They were then subjected to automated amino acid analysis (Applied Biosystems, UK, mod. 420A). The results (Figure 7) indicate the sequence Ile-Glu-Asn-COOH.

Sequences within TBPII were determined by generation of tryptic peptides of the protein. A sample of pure TBPII (200 μg) was reduced, alkylated and repurified on an Aquapore RP-300 reversed phase HPLC column. Fractions containing the modified protein were pooled and the pH was adjusted to 8.0 with $NaHCO_3$. Digestion with TPCK-trypsin (238 U/mg, Millipore Corp., Freehold, NJ) was performed for 16 h at room temperature at an enzyme to substrate ratio of 1:20 (w/w). The digest was loaded onto a C_8 RP-P reversed phase HPLC column (SynChrom, Linden, IN) and the peptides separated by a linear 0–40% acetonitrile gradient in 0.3% aqueous trifluoroacetic acid. The NH_2-terminal amino acid sequences of the peptides and of the intact protein (N-terminus) are presented in Table I. The peptides were numbered according to their sequence of elution from the RP-P column. In the fractions denoted as 39, 44, 46, 53 and 54, where heterogeneity of sequences was observed, both the major and the secondary sequences are presented.

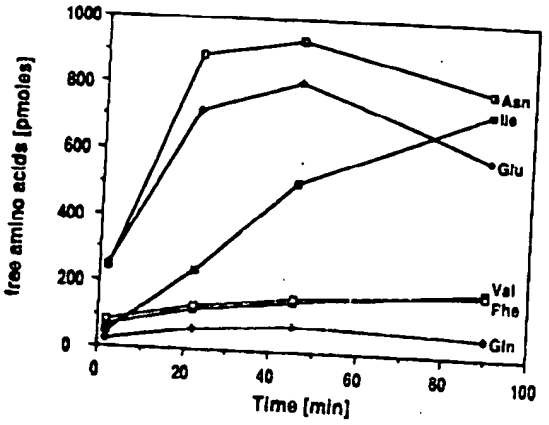

Fig. 7. Time course of the release of COOH-terminal amino acids from TBPI by carboxypeptidase Y.

Isolation of cDNA clones

Three mixtures of synthetic oligonucleotide probes generated from the nucleotide sequence deduced from the NH_2-terminal amino acid sequence of TBPI were used for the screening of cDNA libraries. Initial screenings were carried out with 48-fold degenerate, 26-mers into which deoxyinosine was introduced, wherever the codon ambiguity allowed for all four nucleotides (Figure 1A b). The validity of positive clones was examined by testing their hybridization to two mixed 17-mer nucleotide sequences, containing 96 and 128 degeneracies, corresponding to two overlapping amino acid sequences which constitute part of the sequences to which the 26 bp probes correspond (Figure 1A, c and d). An oligonucleotide probe corresponding to a sequence located close to the 5′ terminus of the longest of the partial cDNA clones isolated with the degenerated probes (nucleotides 478–458 in Figure 1D) was applied for further screening cDNA libraries for a full length cDNA clone. ^{32}P-labeling of the probes, using T4 polynucleotide kinase, plating of the phages in lawns of bacteria, then screening them with the radiolabeled probes, isolation of the positive clones and subcloning of their cDNA inserts were carried out using standard procedures (Sambrook *et al.*, 1989).

Nucleotide sequencing of the cDNA clones

cDNA inserts isolated from positive λGT11 recombinant phages were subcloned into the pBluescript KS(−) vector. Inserts found in λZAP phages were rescued by excising the plasmid pBluescript SK(−) in them, using the R408 helper phage (Short *et al.*, 1988). DNA sequencing in both directions was done by the dideoxy chain termination method (Sanger *et al.*, 1977). Overlapping deletion clones of the cDNAs were generated, in both orientations, by digestion of the cDNA with exonuclease III ('Erase a base' kit, Promega Biotec, Madison, WI). Single-stranded templates derived from these clones using the R408 phage were sequenced with a T7 DNA polymerase sequencing system (Promega.)

Constitutive expression of the type I human TNF-R in CHO cells

The E13 insert was introduced into a modified version of the pSVL expression vector (kindly made available to us by Dr H.Kahana). This construct was transfected, together with the pSV2-DHFR plasmid which contains the DHFR cDNA, into DHFR deficient CHO cells (Chernajovsky *et al.*, 1984), using the calcium phosphate precipitation method (Chen and Okayoma, 1987). Transfection with a recombinant pSVL vector which contained the E13 insert in the inverse orientation served as a control. Cells expressing the DHFR gene were selected by growth in nucleotide-free MEM α medium containing fetal calf serum which had been dialyzed against phosphate buffered saline. Individual clones were picked out and then further selected for amplification of the transfected cDNAs by growth in the presence of 500 nM sodium methotrexate.

Detection of surface-expressed type I TNF-R in the CHO cells

Binding of radiolabeled human rTNF to cells (seeded in 15 mm tissue culture plates at a density of 2.5×10^5 cells/plate) was quantified as described before (Holtmann and Wallach, 1987).

To examine the binding of monoclonal antibodies against TBPI to the CHO cells, the cells were detached by incubation in phosphate buffered saline (PBS: 140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2KPO_4, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$), containing 5 mM EDTA and then incubated for 45 min at 0°C with 50 μg/ml of the test monoclonal antibody in PBS containing 0.5% bovine serum albumin, and 15 mM sodium azide (PBS/BSA). After washing the cells with PBS/BSA they were incubated further for 30 min at 0°C with FITC labeled, affinity purified goat antibody to the F(ab) fragment of mouse IgG (1:120 in PBS/BSA) (Bio-Makor, Israel) and then analyzed by determining the intensity of fluorescence in samples of 10^4 cells using the Becton Dickinson fluorescence activated cell sorter 440. Three monoclonal antibodies to TBPI, clones 17, 18 and 20, shown by cross competition analysis to recognize four spatially distinct epitopes in the TBPI molecule (Engelmann *et al.*, 1990a) and, as a control, a monoclonal antibody against TNF-α (all purified from ascitic fluids by ammonium sulfate precipitation and of the IgG_2 isotype) were used.

Quantification of the soluble form of the type I TNF-R by ELISA

A sensitive enzyme linked immunosorbent assay was set up, using TBPI-specific monoclonal and polyclonal antibodies in a sandwich technique. Immunoglobulins of the anti-TBPI mAb clone 20 (Engelmann *et al.*, 1990a) were adsorbed to 96 well ELISA plates (maxisorp, Nunc, Denmark) by incubation of the plates for 2 h at 37°C with a solution of 25 μg/ml of the antibody in PBS. After incubating the wells further for 2 h at 37°C with a solution containing PBS, 1% BSA, 0.02% NaN_3 and 0.05% Tween 20 (blocking solution) to block nonspecific further binding of protein, tested samples were applied in aliquots of 50 μl/well. The plates were then incubated for 2 h at 37°C, rinsed 3 times with PBS supplemented with 0.05% Tween 20 (washing solution) and then rabbit polyclonal antiserum against TBPI, diluted 1:500 in blocking solution, was added to the wells. After further incubation for 12 h at 4°C, the plates were rinsed again and incubated for 2 h with horse radish peroxidase-conjugated purified goat anti rabbit IgG. The assay was developed using 2,2′-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) as a substrate (Sigma). The enzymatic product was determined colorimetrically at 600 nm. Pure TBPI served as a standard.

Detection of a soluble form of the type I TNF-R in the growth medium of the transfected CHO cells and its analysis by reversed phase HPLC

The amounts of the soluble form of the type I TNF-R in samples of the growth medium of the tested CHO cells, collected 48 h after medium replacement, were determined by the immunoassay described above. For analysis of the soluble receptor by reversed phase HPLC, the CHO cells were cultured for 48 h in serum-free medium (nucleotide-free MEM α). The medium samples were concentrated 100-fold by ultrafiltration on an Amicon PM10 membrane and 100 μl aliquots were then applied to an Aquapore RP300 column (4.6 × 30 mm, Brownlee Labs) pre-equilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with this solution at a flow rate of 0.5 ml/min until all unbound proteins were removed, and then eluted with a concentration gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid, as described before (Engelmann *et al.*, 1989). Fractions of 0.5 ml were collected and, after concentration *in vacuo*, were neutralized with 1 M HEPES buffer, pH 9.0. Amounts of soluble type I TNF-R in the fractions were determined by ELISA and the concentration of protein by the fluorescamine method (Stein and Moschera, 1981).

RNA isolation and analysis

RNA was isolated by a modification of the procedure described by Feramisco *et al.* (Feramisco *et al.*, 1982; Queen and Baltimore, 1983) and analyzed by electrophoresis in 1.5% agarose/6% formaldehyde gels, followed by blotting to 'Genescreen plus' hybridization transfer membranes (NEN, Boston, MA). The E13 cDNA insert was ^{32}P-labeled by random oligomer priming, using the Amersham random primer labeling kit (Amersham, UK). The membranes were hybridized at 42°C in the presence of 50% formamide and then washed as prescribed by Sambrook *et al.* (1989) for the detection of low abundance sequences.

Determination of the effect of TNF and of antibodies to TBPI on protein phosphorylation

Confluent monolayers of HeLa cells (Gey *et al.*, 1952), in 9 mm microwells, were incubated for 100 min with 100 μCi/ml [^{32}P]orthophosphate (Nuclear Research Center, Beer Sheva, Israel) in phosphate-free DMEM containing 10% fetal calf serum which had been dialyzed against 0.9% NaCl. Recombinant human TNF-α (kindly provided by Dr G.Adolf, Boehringer Institute, Vienna, Austria) and rabbit antiserum to TBPI (1:1000) (Engelmann *et al.*, 1990b) or, for comparison, normal rabbit serum (1:1000) were then added to the cells for 20 min. The cells were rinsed, and immediately solubilized by boiling in SDS–PAGE sample buffer containing β-mercaptoethanol. The solubilized proteins were analyzed by SDS–PAGE (12%) followed by

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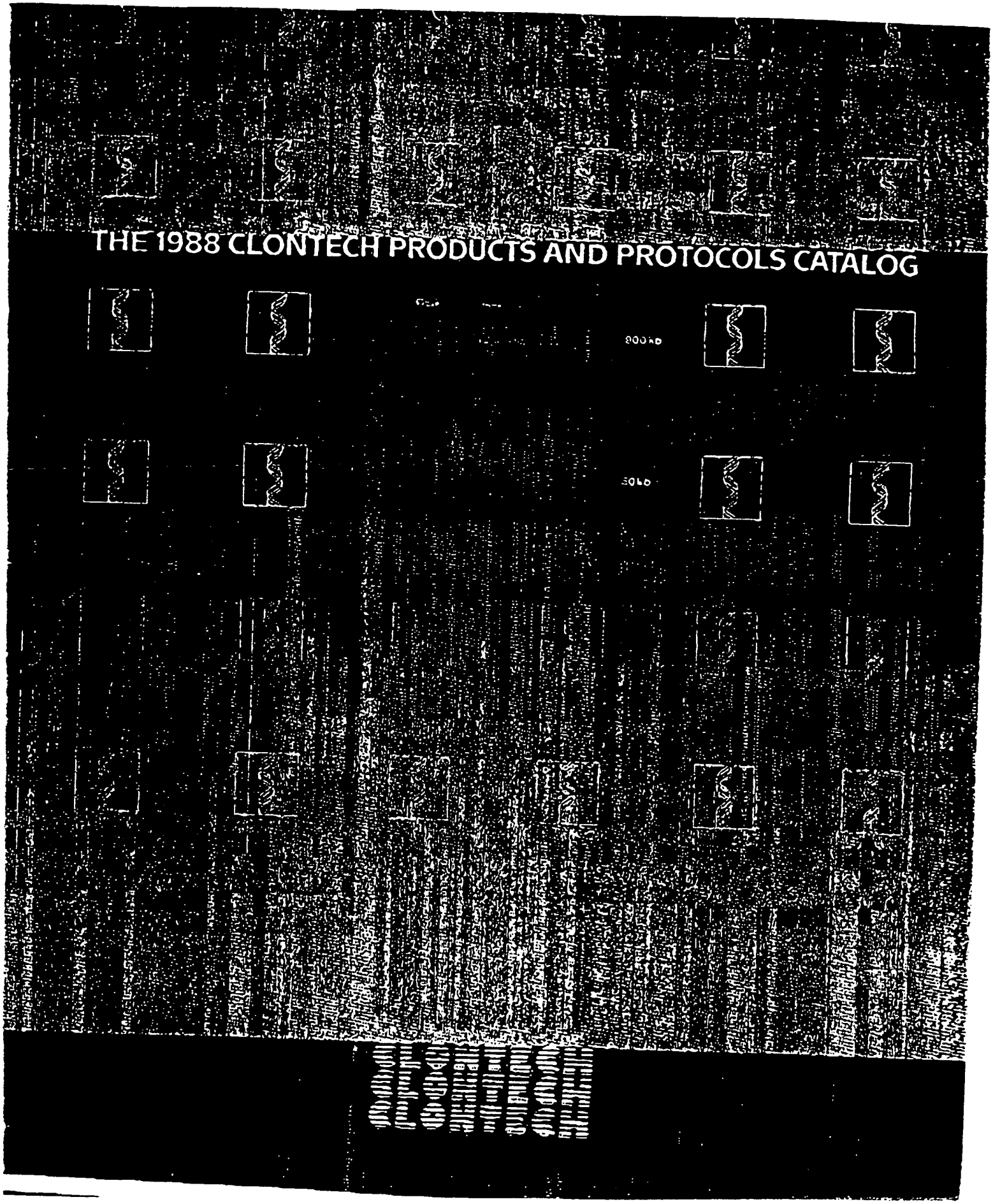
Note added in proof

Since submission of this article, cloning of the cDNA for the type I TNF-R has been described in two publications [Loetscher,H., Pan,Y.-C.E., Lahm,H.W., Gentz,R., Brockhaus,M., Tabuchi,H. and Lesslauer,W. (1990) *Cell*, **61**, 351–359 and Schall,T.J. *et al.*, *ibid.*, 361–370.] In two other publications [Smith,C.A. *et al.* (1990) *Science*, **248**, 1019–1023 and Kohno,T. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* (in press)] the cloning of the type II TNF-R was described. The predicted amino acid sequence in the extracellular domain of this receptor fully matches the sequences presented here of amino acids in TBP II indicating further that, like TBPI, this soluble TNF binding protein is also derived from its immunologically cross-reacting cell surface TNF-R.

Appendix F



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Human Libraries					
Human Adrenal cDNA	λgt10	Cortex and Medulla of adult with Cushing's Disease.	1.3×10^6	0.55–3.3 kb (1.2 kb)	HL1014a
Human AML cDNA	λgt11	Acute myelogenous leukemia (KG-1).	1.4×10^6	0.8–3.4 kb (1.3 kb)	HL1046b
Human B-Cell cDNA	λgt10	B-Cell leukemic cell line, RPMI 4265. Turner, *et al., J. Biol. Chem.*, 250: 4512 (1975).	1.3×10^6	0.39–3.4 kb (0.98 kb)	HL1018a
Human B-Cell cDNA	λgt11	B-Cell leukemic cell line, RPMI 4265. Turner, *et al., J. Biol. Chem.*, 250: 4512 (1975).	8.9×10^5	0.73–4.1 kb (1.2 kb)	HL1018b
Human B-Cell, PMA activated, cDNA	λgt10	RPMI 4265 cells treated with 50 ng/ml Phorbolmyristate acetate PMA for 4 days.	1.4×10^6	0.6–3.7 kb (1.2 kb)	HL1035a
Human Brain cDNA	λgt11	Normal temporal cortex tissue, excised from around a tumor.	7.3×10^6	0.4–3.1 kb (780 bp)	HL1003b
Human Brain, Alzheimer's, cDNA	λgt11	Tissue around the hippocampus of a 70-year old diseased female, removed 5-8 hours after death.	8.1×10^5	0.31–3.1 kb (1.1 kb)	HL1028b
Human CML Spleen cDNA	λgt11	Spleen of eight year old female with Chronic Myelogenous Leukemia.	1.4×10^6	0.5–3.8 kb (1.1 kb)	HL1040b
Human Colon cDNA	λgt10	Normal tissues excised from around the colon cancer of a 53 year old male.	1.5×10^6	0.6–3.2 kb (0.9 kb)	HL1034a
Human Colon cDNA	λgt11	Normal tissues excised from around the colon cancer of a 53 year old male.	1.3×10^6	0.6–3.5 kb (1.1 kb)	HL1034b
Human Endothelial cDNA	λgt11	Endothelial cells from umbilical cord veins. Cells were serially passaged and poly(A) isolated.	1.5×10^6	0.34–3.4 kb (0.9 kb)	HL1024b
Human Epithelial cDNA	λgt11	Normal thymus epithelial cells, excised during open-heart surgery on a 3 year old Caucasian female.	1.4×10^6	0.42–2.8 kb (0.9 kb)	HL1025b

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Erythroleukemic cDNA	λgt10	Human erythroleukemic K562 cells cultured in RPMI 1640. Siebert and Fukuda, *J. Biol. Chem.*, 260: 640 (1985). Lozzio and Lozzio, *Blood*. 45: 321 (1975).	1.0×10^6	0.5–3.4 kb (1.0 kb)	HL1032a
Human Eye cDNA	λ gt10	Male/female eye pool.	1.1×10^6	0.4–3.2 kb (0.7 kb)	HL1047a
Human Eye cDNA	λ gt11	Male/female eye pool.	1.5×10^6	0.4–2.8 kb (0.7 kb)	HL1047b
Human Fibroblast, Lung, cDNA	λgt11	Lung fibroblast cell line, IMR-90. ATCC# CCL186.	2.2×10^6	0.5–3.0 kb (1.3 kb)	HL1011b
Human Fibroblast, Skin, cDNA	λ gt10	Cultured primary fibroblasts from a young male.	1.4×10^6	0.5–3.6 kb (1.0 kb)	HL1052a
Human Fibroblast, Skin, cDNA	λ gt11	Cultured primary fibroblasts from a young male.	1.1×10^6	0.5–3.5 kb (1.1 kb)	HL1052b
Human Fibrosarcoma cDNA	λ gt11	HT-1080 tumor cells. Rasheed *et al., Cancer* 33:1027 (1974). ATCC # CCL121.	1.6×10^6	1.0–3.4 kb (1.3 kb)	HL1048b
Human Glioma cDNA	λ gt11	HS 683 from explant cultures of a glioma taken from the left temporal lobe. ATCC # HTB 138.	1.1×10^6	0.4–3.1 kb (0.9 kb)	HL1049b
Human Hairy Cell Leukemia (Mo-B) cDNA	λgt10		1.4×10^6	0.4–3.2 kb (1.0 kb)	HL1043a
Human Heart cDNA	λgt11	Adult male heart, including some aorta region. Poly(A) RNA was slightly degraded as visualized on alkaline agarose gel.	1.27×10^6	0.4–3.4 kb (0.9 kb)	HL1038b
Human Heart, Fetal Aorta, cDNA	λgt11	Normal, third trimester heart tissue.	8.4×10^5	0.5–3.5 kb (1.1 kb)	HL1042b
Human HeLa Cell cDNA	λgt11	HeLa-derived D98-AH2 cells, HPRT- in phenotype. W.S. Szybalski *et al., Natl. Cancer Institute Monograph*, 7: 75 (1962).	1.3×10^6	0.48–3.1 kb (0.86 kb)	HL1022b
Human Hepatoma cDNA	λgt11	Hepatoma cells, G2.	1.3×10^6	0.5–3.7 kb (0.96 kb)	HL1015b

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Keratinocyte cDNA	λgt11	Primary keratinocyte culture from adult epidermis.	1.7 X 10^6	0.5–3.8 kb (1.1 kb)	HL1045b
Human Kidney cDNA	λgt10	From a juvenile male whose kidney had been perfused for 24 hours prior to poly(A) RNA isolation.	1.7 X 10^6	0.5–3.2 kb (0.9 kb)	HL1033a
Human Kidney cDNA	λgt11	From a juvenile male whose kidney had been perfused for 24 hours prior to poly(A) RNA isolation.	1.4 X 10^6	0.4–3.1 kb (0.9 kb)	HL1033b
Human Leukocyte Genomic	EMBL3	Leukocyte genomic DNA, male.	8.8 X 10^5	16kb	HL1006d
Human Leukocyte, Peripheral Blood, cDNA	λgt10	Peripheral blood leukocytes from adult female with Acute Promyelocytic Leukemia, HL60. S.J.Collins *et al.*, *Nature*, 270: 347 (1977). ATCC# CCL240.	7.2 X 10^5	0.69–3.5 kb (0.88 kb)	HL1020a
Human Leukocyte, Peripheral Blood, cDNA	λgt11	Peripheral blood leukocytes from adult female with Acute Promyelocytic Leukemia, HL60. S.J.Collins *et al.*, *Nature*, 270: 347 (1977). ATCC# CCL240.	8.4 X 10^5	0.77–3.6 kb (1.1 kb)	HL1020b
Human Liver cDNA	λgt10	Normal adult liver, female.	1.5 X 10^5	0.17–2.4 kb (0.8 kb)	HL1001a
Human Liver cDNA	λgt11	Normal adult liver, female.	7.8 X 10^5	0.3–2.9 kb (0.94 kb)	HL1001b
Human Liver, Fetal, cDNA	λgt11	Fetal liver, 1st trimester, male.	2.3 X 10^5	0.14–2.3 kb (1.1 kb)	HL1005b
Human Lung cDNA	λgt11	Normal adult lung tissue, excised during surgery.	8.2 X 10^5	0.25–3.1 kb (1.2 kb)	HL1004b
Human Lung Fibroblast cDNA	λgt11	Lung fibroblast cell line, IMR-90. ATCC# CCL186.	2.2 X 10^6	0.5–3.0 kb (1.3 kb)	HL1011b
Human Lung Small Cell Carcinoma (NCI-H69) cDNA	λgt10	Lung Small Cell Carcinoma cell culture, NCI-H69.	1.4 X 10^6	0.6–3.6 kb (0.93 kb)	HL1012a
Human Lung Small Cell Carcinoma (NCI-H69) cDNA	λgt11	Lung Small Cell Carcinoma cell culture, NCI-H69.	7.7 X 10^5	0.4–2.8 kb (1.0 kb)	HL1012b

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Lung Small Cell Carcinoma (NCI-H128) cDNA	λgt10	From NCI-H128 cell line derived from malignant pleural fluid of a 60 year old Black male. Gazder, *et al.*, *Cancer Research*, 40: 3502 (1980).	1.0×10^6	0.3–3.5 kb (0.85 kb)	HL1030a
Human Lung Small Cell Carcinoma (NCI-H128) cDNA	λgt11	From NCI-H128 cell line derived from malignant pleural fluid of a 60 year old Black male. Gazder, *et al.*, *Cancer Research*, 40: 3502 (1980).	1.3×10^6	0.3–3.7 kb (0.92 kb)	HL1030b
Human Lung, WI-38, cDNA	λgt11	WI-38 human diploid cell line, derived from normal Caucasian female lung. L Hayflick, *Exp. Cell Res.*, 25: 585 (1961).	1.2×10^6	0.6–3.5 kb (1.1 kb)	HL1041b
Human Lymphocyte cDNA	λgt10	Near confluent Raji cells, B lymphocytes. Pulvertaft, *Lancet*, 1: 238 (1964). ATCC# CCL86.	1.5×10^6	0.25–3.2 kb (0.87 kb)	HL1002a
Human Mammary Gland cDNA	λgt10	Adult female breast tissue excised during mastectomy. The female was in the 8th month of pregnancy, showing well-differentiated tissues and lactational competence.	1.1×10^6	0.6–3.3 kb (1.1 kb)	HL1037a
Human Mammary Gland cDNA	λgt11	See above.	1.1×10^6	0.5–3.4 kb (1.2 kb)	HL1037b
Human Melanoma cDNA	λgt11	Near confluent melanoma A2058 cells.	7.1×10^5	0.3–3.6 kb (0.82 kb)	HL1023b
Human Monocyte, Peripheral Blood, cDNA	λ gt 10	90% human peripheral blood monocytes, LPS-activated.	1.34×10^6	0.4–3.5 kb (1.2 kb)	HL1050a
Human Monocyte, Peripheral Blood, cDNA	λ gt11	90% human peripheral blood monocytes, LPS-activated.	1.3×10^6	0.5–3.7 kb (1.0 kb)	HL1050b
Human Monocyte, THP-1, cDNA	λgt10	THP-1 monocytes from 1 year old male with Acute Monocytic leukemia. *Int. J. Cancer*, 26: 171 (1980). *Cancer Research*, 42: 1530 (1982). ATCC# TIB202.	1.4×10^6	0.65–3.0 kb (0.97 kb)	HL1021a

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Monocyte, U-937, cDNA	λgt10	U-937 cultured cells actively growing prior to poly(A) RNA isolation.	1.5×10^6	0.4–3.4 kb (0.95 kb)	HL1029a
Human Monocyte, U-937, cDNA	λgt11	U-937 cultured cells actively growing prior to poly(A) RNA isolation.	1.2×10^6	0.3–3.7 kb (0.95 kb)	HL1029b
Human Monocyte, U-937, PMA activated cDNA	λgt10	U-937 cells treated with 50 ng/ml Phorbolmyristate acetate (PMA) for 3.5 days to achieve monocyte-like stage.	1.4×10^6	0.6–3.8 kb (1.1 kb)	HL1036a
Human Monocyte, U-937, PMA activated cDNA	λgt11	U-937 cells treated with 50 ng/ml Phorbolmyristate acetae (PMA) for 3.5 days to achieve monocyte-like stage.	1.2×10^6	1.0–3.9 kb (1.3 kb)	HL1036b
Human Multiple Myeloma cDNA	λgt11	Bone marrow obtained from a female with Multiple Myeloma, IM9. *Ann. N.Y. Acad. Sci.*, 190: 221 (1972). *PNAS*, 71: 84 (1974). *J. Biol. Chem.*, 249: 1661 (1974). ATCC# CCL159.	2.2×10^6	0.52–3.5 kb (1.1 kb)	HL1027b
Human Neuroblastoma cDNA	λgt10	Neuroblastoma cell line, Kelly.	1.05×10^5	0.4–3.4 kb (1.3 kb)	HL1007a
Human Osteosarcoma cDNA	λgt11	Osteosarcoma cell culture, MG-63. *Antimicrob. Ag. Chemother.*, 12: 11 (1977). ATCC# CRL1427	1.6×10^6	0.55–3.0 kb (0.97 kb)	HL1013b
Human Pancreas cDNA	λgt10	Islets of Langerhans	1.0×10^6	1.2–4.0 kb (2.0 kb)	HL1054a
Human Peripheral Blood Leukocyte cDNA	λgt10	Peripheral blood leukocytes from adult female with Acute Promyelocytic Leukemia, HL60. S.J.Collins *et al.*, *Nature*, 270: 347 (1977). ATCC# CCL240.	7.2×10^5	0.69–3.5 kb (0.88 kb)	HL1020a
Human Peripheral Blood Leukocyte cDNA	λgt11	Peripheral blood leukocytes from adult female with Acute Promyelocytic Leukemia, HL60. S.J.Collins *et al.*, *Nature*, 270: 347 (1977). ATCC# CCL240.	8.4×10^5	0.77–3.6 kb (1.1 kb)	HL1020b
Human Peripheral Blood Monocyte cDNA	λ gt10	90% human peripheral blood monocytes, LPS-activated.	1.34×10^6	0.4–3.5 kb (1.2 kb)	HL1050a

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Peripheral Blood Monocyte cDNA	λ gt 11	90% human peripheral blood monocytes, LPS-activated.	1.3 X 10^6	0.5–3.7 kb (1.0 kb)	HL1050b
Human Peripheral Blood Monocyte cDNA	λ gt 11	90% human peripheral blood monocytes.	1.2 X 10^6	0.5–3.4 kb (1.1 kb)	HL1056b
Human Placenta cDNA	λgt11	Placental tissue, 34 weeks old.	1.0 X 10^6	0.8–3.6 kb (1.8 kb)	HL1008b
Human Prostate cDNA	λ gt10	Normal 65 year old prostate tissues.	1.5 X 10^6	0.6–3.7 kb (1.2 kb)	HL1051a
Human Prostate cDNA	λ gt11	Normal 65 year old prostate tissues.	1.62 X 10^6	0.5–3.6 kb (1.1 kb)	HL1051b
Human Retina cDNA	λgt10	Adult retina.	1.6 X 10^6	0.5–3.5 kb (1.1 kb)	HL1055a
Human Retina cDNA	λgt11	Adult retina.	1.4 X 10^6	0.5–3.4 kb (1.0 kb)	HL1055b
Human Skin Fibroblast cDNA	λ gt10	Cultured primary fibroblasts from a young male.	1.4 X 10^6	0.5–3.6 kb (1.0 kb)	HL1052a
Human Skin Fibroblast cDNA	λ gt11	Cultured primary fibroblasts from a young male.	1.1 X 10^6	0.5–3.5 kb (1.1 kb)	HL1052b
Human Spleen cDNA	λgt10	Normal 25 year old male spleen.	1.4 X 10^6	0.6–3.4 kb (1.0 kb)	HL1039a
Human Spleen cDNA	λgt11	Normal 25 year old male spleen.	1.3 X 10^6	0.6–3.3 kb (1.0 kb)	HL1039b
Human Spleen, CML, cDNA	λgt11	Spleen of 8 year old female with Chronic Myelogenous Leukemia.	1.4 X 10^6	0.5–3.8 kb (1.1 kb)	HL1040b
Human Submaxillary Gland cDNA	λ gt11	Male gland excised during biopsy.	1.3 X 10^6	0.5–3.2 kb (1.0 kb)	HL1053b
Human T-Cell cDNA	λgt10	T-Cell leukemic cell line. Jurkat, Schneider *et al., Int. J. Cancer*, 19:621 (1977). Yanagi *et al., PNAS*, 82: 3430 (1985).	1.1 X 10^6	0.58–3.8 kb (1.2 kb)	HL1016a
Human T-Cell cDNA	λgt11	T-Cell Leukemic cell line. Jurkat, Schneider *et al., Int. J. Cancer*, 19:621 (1977). Yanagi *et al., PNAS*, 82: 3430 (1985).	1.1 X 10^6	0.58–3.8 kb (1.2 kb)	HL1016b

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human T-Cell, PHA Stimulated, cDNA	λgt10	Concentrated T-Cell population from peripheral blood of a healthy adult. PHA stimulated for 48 hours.	1.5×10^6	0.45–3.8 kb (0.95 kb)	HL1031a
Human T-Cell, PHA Stimulated, cDNA	λgt11	Concentrated T-Cell population from peripheral blood of a healthy adult. PHA stimulated for 48 hours.	1.0×10^6	0.7–3.3 kb (1.4 kb)	HL1031b
Human Testis cDNA	λgt11	Normal testicle, excised during surgery from a healthy 50 year old. Not hormonally induced.	1.0×10^6	0.7–3.3 kb (1.2 kb)	HL1010b
Human Thymocyte DNA	λgt11	Normal thymus excised during surgery on a 3 year old Caucasian female.	1.7×10^6	0.45–3.4 kb (0.9 kb)	HL1026b
Human Thyroid Carcinoma cDNA	λgt11	Adult thyroid carcinoma tissue, male.	6.8×10^5	0.65–2.7 kb (1.2 kb)	HL1009b
Human Wilms' Tumor (G-401) cDNA	λgt11		1.3×10^5	0.6–3.8 kb	HL1044b

Monkey Libraries

Monkey Brain cDNA	λgt10	Adult male Rhesus brain tissue.	1.3×10^6	0.5–3.4 kb	OL1003a
Monkey Brain cDNA	λgt11	Adult male Rhesus brain tissue.	1.2×10^5	0.6–3.7 kb	OL1003b
Monkey Genomic	EMBL3	Adult female Rhesus liver.	1.6×10^6	10–22 kb (16.3 kb)	OL1004d
Monkey Liver cDNA	λgt10	Adult male Rhesus liver tissue.	1.3×10^6	0.5–3.4 kb	OL1002a
Monkey Liver cDNA	λgt11	Adult male Rhesus liver tissue.	1.1×10^6	0.6– 3.5 kb	OL1002b
Monkey Smooth Muscle cDNA	λgt10	Aortic smooth muscles from 1.5 year old Rhesus.	1.4×10^6	0.4–3.8 kb (1.03 kb)	OL1001a
Monkey Smooth Muscle cDNA	λgt11	Aortic smooth muscles from 1.5 year old Rhesus.	1.3×10^6	0.5–3.3 kb (1.1 kb)	OL1001b

Appendix G

DA39

Tools For the Molecular Biologist (1989/1990)

Table of Contents

Table of Contents

CLONTECH Ordering Information

Technical Information:

Our scientific staff is happy to assist you with any questions concerning the use of our products.

CLONTECH is committed to providing you with the best innovative products and services possible. We value you as a customer. If you are dissatisfied in any way with our products or services, please let us know.

We are easy to reach between the hours of 8 AM and 6 PM, Monday through Friday at:

(415) 424-8222

or, you may contact us through your distributor.

How to order:

Outside the United States:

CLONTECH's distributors around the world are pleased to accept your orders for their countries of license. Please refer to the distributor listing in this appendix for contact information. If you are ordering directly from Canada, please supply the name and telephone number of your Customs broker.

Within the United States:

To expedite your order, please provide the following information:

1. Shipping address
2. Billing address
3. Purchase order number
4. Catalog number and product description
5. Size and quantity of product
6. Purchaser's name and contact number

Orders are processed upon receipt, in most cases within 24 hours for routinely inventoried products.

To avoid duplication of your order, please make sure that written confirming orders are clearly marked as such. CLONTECH does not require written confirmation of phone orders. If you receive duplicate shipments, please contact us immediately

Telephone orders:

Call (800) 662-CLON (outside CA)

or (415) 424-8222 (inside CA)

Fax orders:

Our fax number is: (415) 424-1352

Telex orders:

Our telex number is: 330060

Mail orders:

Please send your ordering information to:

CLONTECH Laboratories, Inc.
4030 Fabian Way
Palo Alto, CA 94303
Attention: Customer Service Department

Standing Orders:

CLONTECH offers periodic shipments on any purchase order as a service to our frequent customers. Please arrange a schedule with your customer service representative.

Custom and Bulk Orders:

Please request quotations for all custom and bulk orders from your Customer Service Representative.

Minimum Order:

CLONTECH has no minimum order quantity. There is a $5.00 handling charge for orders under $150.00.

CLONTECH Ordering Information

Pricing and Terms:

Prices are subject to change without notice. For the most up-to-date pricing information on items in this price listing, please contact us or your distributor.

In the event of interim price changes on products amounting to more than 10% on orders over $100.00, **CLONTECH** will contact you prior to shipping your order. Payment terms are net 30 days in US dollars.

US Government Customers

CLONTECH is listed on the GSA Schedule. Our NIH BPA Number is 263-00025536.

Shipping:

CLONTECH products are shipped F.O.B. Palo Alto, CA. Shipping charges are prepaid and added to your invoice. Orders requiring blue or dry ice are shipped via UPS Next Day Air or Federal Express P1, Monday through Thursday, unless otherwise requested. Orders not requiring ice are shipped via UPS 2 Day Air, Monday through Friday. Shipments of hazardous items are sent by an appropriate carrier.

Alternative carriers may be requested with your order.

Partial Shipments

Normally, all orders may be filled within a single shipment. If this is not possible, we will initiate a partial shipment of your order. If you do not want a partial shipment, please indicate this when you order.

Returns and Credit:

In order to facilitate processing, we request that no returns be made without prior authorization. Please contact your customer service representative for instructions for all returned goods. In order to receive credit, products should be returned in original, intact condition. There will be a 20% restocking fee on all goods returned due to customer ordering error.

Conditions:

All of **CLONTECH**'s products are intended to be used only in a research laboratory. They are not to be used either for drug or diagnostic purposes, nor are they intended to be for human use.

Product Changes:

We reserve the right to delete products or change specifications at any time without notice.

Genetic Research Guidelines:

We recommend that our products be used in accordance with NIH guidelines developed for genetic research.

Patent Information:

No license or immunity under any patent is either granted or implied by the sale of any of our products.

Disclaimer:

CLONTECH disclaims any and all responsibility for any injury or damage which may be caused by the failure of the buyer or any other person to use these products in accordance with the conditions outlined herein.

Warranty:

CLONTECH's products are warranted to meet our product specifications in effect at the time of shipment. Notice of non-conforming products must be made to **CLONTECH** within 30 days of receipt of the product. This warranty is exclusive and is in lieu of all other warranties, expressed or implied, including any implied warranty of merchantability or fitness for any particular purpose. **CLONTECH** shall not be liable for any incidental, consequential or contingent damages.

Pricing and Terms:

Prices are subject to change without notice. For the most up-to-date pricing information on items in this price listing, please contact us or your distributor.

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US Government Customers

CLONTECH is listed on the GSA Schedule. Our VA contract number is V797P-5624K. Our NIH BPA Number is 263-00025536.

Shipping:

CLONTECH products are shipped F.O.B. Palo Alto, CA. Shipping charges are prepaid and added to your invoice. Orders requiring wet or dry ice are shipped via UPS Next Day Air or Federal Express P1, Monday through Thursday, unless otherwise requested. Orders not requiring ice are shipped via UPS 2 Day Air, Monday through Friday. Shipments of hazardous items are sent by an appropriate carrier.

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Normally, all orders may be filled within a single shipment. If this is not possible, we will initiate a partial shipment of your order. If you do not want a partial shipment, please indicate this when you order.

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We recommend that our products be used in accordance with NIH guidelines developed for genetic research.

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No license or immunity under any patent is either granted or implied by the sale of any of our products.

Disclaimer:

CLONTECH disclaims any and all responsibility for any injury or damage which may be caused by the failure of the buyer or any other person to use these products in accordance with the conditions outlined herein.

Warranty:

CLONTECH's products are warranted to meet our product specifications in effect at the time of shipment. Notice of non-conforming products must be made to **CLONTECH** within 30 days of receipt of the product. This warranty is exclusive and is in lieu of all other warranties, expressed or implied, including any implied warranty of merchantability or fitness for any particular purpose. **CLONTECH** shall not be liable for any incidental, consequential or contingent damages.

1989 – 1990

CLONTECH Libraries

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Hamster Libraries					
CHO-K1 cDNA, 5'-STRETCH	λgt10	Chinese hamster ovary CHO-K1 cells. ATCC#CCL61.	1.4×10^6	0.6 to>4.0 kb (1.5 kb)	JL1001a
[illegible] cDNA	λgt11	Chinese hamster ovary CHO-K1 cells. ATCC#CCL61.	1.5×10^6	0.7 to>4.0 kb (1.5 kb)	JL1001b
CHO-K1 cDNA, 5'-STRETCH	SWAJ-2	See above.	1.4×10^6	0.6 to>4.0 kb (1.1 kb)	JL1001h
Human Libraries					
[illegible]al cDNA	λgt10	Cortex and Medulla of adult with Cushing's Disease.	1.3×10^6	0.55–3.3 kb (1.2 kb)	HL1014a
[illegible]man AML cDNA	λgt11	Acute myelogenous leukemia(KG-1).	1.4×10^6	0.8–3.4 kb (1.3 kb)	HL1046b
Human, Aorta, Fetal, cDNA	λgt11	Normal, third trimester, male aortal tissue.	8.4×10^5	0.5–3.5 kb (1.1 kb)	HL1042b
[illegible] cDNA	λgt10	B-Cell leukemic cell line, RPMI 4265. Turner, *et al., J. Biol. Chem.*, 250: 4512 (1975).	1.3×10^6	0.39–3.4 kb (0.98 kb)	HL1018a
Human B-Cell cDNA	λgt11	See above.	8.9×10^5	0.73–4.1 kb (1.2 kb)	HL1018b
Human B-Cell, PMA [illegible]ated, cDNA	λgt10	RPMI 4265 cells treated with 50 ng/ml Phorbolmyristate acetate PMA for 4 days.	1.4×10^6	0.6–3.7 kb (1.2 kb)	HL1035a
Human Bone Marrow, 5'-STRETCH cDNA	λ gt10	Adult male.	1.4×10^6	0.6 to>4.0 kb (1.5 kb)	HL1058a
Human Bone Marrow, 5'-STRETCH cDNA	λ gt11	Adult male.	1.51×10^6	0.6 to>4.0 kb (1.6 kb)	HL1058b
Human Brain, Amygdala cDNA	λgt10		1.6×10^6	0.6–3.5 kb (1.2 kb)	HL1080a
[illegible] Brain, Amygdala cDNA	λgt11		1.3×10^6	0.6–3.7 kb (1.2 kb)	HL1080b
Human Brain, Calcarine Cortex cDNA	λgt10		1.4×10^6	0.6–3.5 kb (1.0 kb)	HL1081a
Human Brain, Calcarine Cortex cDNA	λgt11		1.0×10^6	0.6–3.8 kb (1.4 kb)	HL1081b

CLONTECH Libraries

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Brain, Caudate cDNA	λgt10		1.8×10^6	0.6–3.8 kb (1.4 kb)	HL1076a
Human Brain, Caudate cDNA	λgt11		1.5×10^6	0.6–3.8 kb (1.4 kb)	HL1076b
Human Brain, Cerebellar Vermis cDNA	λgt10		1.8×10^6	0.6–3.7 kb (1.3 kb)	HL1083a
Human Brain, Cerebellar Vermis cDNA	λgt11		1.2×10^6	0.6–3.6 kb (1.2 kb)	HL1083b
Human Brain, Cingulate Gyrus cDNA	λgt10		1.5×10^6	0.5–3.4 kb (1.0 kb)	HL1084a

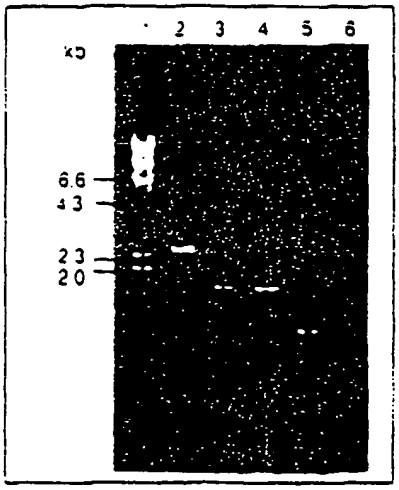


Figure 2. Region-Specific Human Brain Libraries. These libraries are made from oligo(dT)-primed and randomly-primed cDNA from specific regions of the human brain. The tissues were obtained at autopsy from a disease-free 20 year old black male. The body was preserved at 4°C within one hour of death. Shown here are insert sizes of four random clear plaques from the λgt11 gyrus recti library, determined by using two λgt11 GeneAmp™ PCR™ PRI-MATE™ Amplimers (Cat No. 5412-1).

Human Brain, Cingulate Gyrus cDNA	λgt11		1.2×10^6	0.5–3.5 kb (1.1 kb)	HL1084b
Human Brain, Dentate cDNA	λgt10		1.4×10^6	0.4–3.5 kb (1.0 kb)	HL1085a
Human Brain, Dentate cDNA	λgt11		1.0×10^6	0.4–3.5 kb (1.1 kb)	HL1085b
Human Brain, Globus Pallidus cDNA	λgt10		1.2×10^6	0.5–4.0 kb (1.2 kb)	HL1086a
Human Brain, Globus Pallidus cDNA	λgt11		1.2×10^6	0.5–3.7 kb (1.1 kb)	HL1086b
Human Brain, Gyrus Recti cDNA	λgt10		1.5×10^6	0.6–4.0 kb (1.5 kb)	HL1087a
Human Brain, Gyrus Recti cDNA	λgt11		1.3×10^6	0.6–4.0 kb (1.6 kb)	HL1087b

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Brain, Hippocampus cDNA	λgt10		1.0×10^6	0.3–3.2 kb (1.0 kb)	HL1088a
Human Brain, Hippocampus cDNA	λgt11		1.1×10^6	0.3–3.2 kb (0.8 kb)	HL1088b
Human Brain, Medulla	λgt10		1.3×10^6	0.4–3.8 kb (1.0 kb)	HL1089a
Human Brain, Medulla cDNA	λgt11		1.2×10^6	0.4–3.7 kb (1.1 kb)	HL1089b
Human Brain, Nucleus Accumbens cDNA	λgt10		1.0×10^6	0.5–3.7 kb (1.2 kb)	HL1090a
Human Brain, Nucleus Accumbens cDNA	λgt11		1.1×10^6	0.6–3.8 kb (1.1 kb)	HL1090b
Human Brain, Occipital Pole cDNA	λgt10		1.8×10^6	0.5–3.4 kb (1.1 kb)	HL1091a
Human Brain, Occipital Pole cDNA	λgt11		1.6×10^6	0.5–3.5 kb (1.1 kb)	HL1091b
Human Brain, Putamen cDNA	λgt10		1.3×10^6	0.4–3.3 kb (0.9 kb)	HL1092a
Human Brain, Putamen cDNA	λgt11		1.3×10^6	0.4–3.4 kb (0.9 kb)	HL1092b
Human Brain, Substantia Nigra cDNA	λgt10		1.8×10^6	0.6–4.0 kb (1.4 kb)	HL1093a
Human Brain, Substantia Nigra cDNA	λgt11		1.2×10^6	0.6–4.0 kb (1.4 kb)	HL1093b
Human Brain, Superior Temporal Gyrus cDNA	λgt10		1.0×10^6	0.6–3.7 kb (1.1 kb)	HL1094a
Human Brain, Superior Temporal Gyrus cDNA	λgt11		1.0×10^6	0.4–3.7 kb (1.2 kb)	HL1094b
Human Brain, Fetal, cDNA	λgt10	Fetal Brain tissue from 21 week old fetus. Mother's blood type: A+.	1.3×10^6	0.6–4.0 kb (1.1 kb)	HL1065a
Human Brain, Fetal, cDNA	λgt11	See above.	1.3×10^6	0.7–4.0 kb (1.2 kb)	HL1065b
Human Brain, Temporal Cortex cDNA	λgt11	Normal temporal cortex tissue, excised from around a tumor.	7.3×10^5	0.4–3.1 kb (780 bp)	HL1003b

CLONTECH Libraries

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Brain, Alzheimer's, cDNA	λgt11	Tissue around the hippocampus of a 70-year old diseased female, removed 5-6 hours after death.	8.1×10^5	0.31–3.1 kb (1.1 kb)	HL1028b
Human Breast cDNA	λgt10	Adult breast tissue excised during mastectomy, during the 8th month of pregnancy. Section showed well-differentiated tissue and lactational competence.	1.1×10^6	0.6–3.3 kb (1.1 kb)	HL1037a
Human Breast cDNA	λgt11	See above.	1.6×10^6	0.5–3.4 kb (1.2 kb)	HL1037b
Human Breast, 5′-STRETCH cDNA	λgt10	See above.	1.4×10^5	0.8 to>4.0 kb (1.5 kb)	HL1061a
Human Breast, 5′-STRETCH cDNA	λgt11	See above.	1.3×10^6	0.8 to>4.0 kb (1.3 kb)	HL1061b
Human Breast, 5′-STRETCH cDNA	SWAJ-2	See above.	1.4×10^6	0.8 to>4.0 kb (1.5 kb)	HL1061h
Human Breast Carcinoma, 5′-STRETCH cDNA	λ gt10	Breast carcinoma ZR-75-1.	1.3×10^6	1.0 to>4.0 kb (1.7 kb)	HL1059a
Human Breast Carcinoma, 5′-STRETCH cDNA	λ gt11	Breast carcinoma ZR-75-1.	1.4×10^6	1.0 to>4.0 kb (1.7 kb)	HL1059b
Human CEM T-Cell cDNA, randomly-primed + oligo(dT)-primed	Zap†	CCRF-CEMT lymphoblastoid cell line (Foley *et al.*, *Cancer* 18: 522 (1965).) Cell line is positive for CD4, CD5, T9 and T10, and negative for CD1, CD3, CD8, DR and TAC. Cell line is used for the propagation of HIV. ATCC # CCL 119.	1.6×10^6	1.2 to >4.0 kb (1.7 kb)	HL1063f
Human CEM T-Cell cDNA, randomly-primed + oligo(dT)-primed	pBlue-script†	See above.	1.0×10^6	1.1–4.6 kb (2.3 kb)	HL1063g
Human CML Spleen cDNA	λgt11	Spleen of eight year old female with Chronic Myelogenous Leukemia.	1.4×10^6	0.5–3.8 kb (1.1 kb)	HL1040b

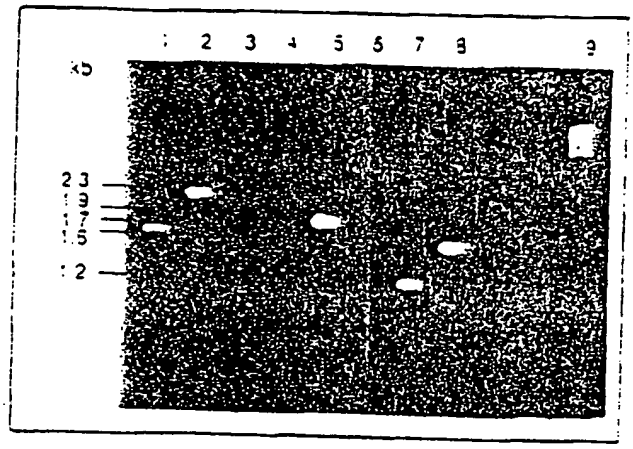
L	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Colon cDNA	λgt10	Normal tissues excised from around the colon cancer of a 53 year old male.	1.5×10^6	0.6–3.2 kb (0.9 kb)	HL1034a
Human Colon cDNA	λgt11	See above.	1.3×10^6	0.6–3.5 kb (1.1 kb)	HL1034b
[illegible] Colon Tumor	λgt11 *Not I/Sfi*	Human T84 colonic tumor cell line.	1.3×10^6	0.5–3.5 kb (1.1 kb)	HL1079k
Human Endothelial cDNA	λgt11	Endothelial cells from umbilical cord veins. Cells were serially passaged and poly(A) isolated.	1.5×10^6	0.34–3.4 kb (0.9 kb)	HL1024b
Human Endothelial cDNA, 5′-STRETCH	λgt11	Endothelial cells cultured from human umbilical cord veins.	2.1×10^6	0.6 to >4.0 kb (1.8 kb)	HL1070b
[illegible] Epithelial cDNA	λgt11	Normal thymus epithelial cells, excised during open-heart surgery on a 3 year old Caucasian female.	1.4×10^6	0.42–2.8 kb (0.9 kb)	HL1025b
Human Erythroleukemic cDNA	λgt10	Human erythroleukemic K562 cells cultured in RPMI 1640 and treated with PMA as described. Siebert and Fukuda, *J. Biol. Chem.*, 260: 640 (1985). Lozzio and Lozzio, *Blood*, 45: 321 (1975).	1.0×10^6	0.5–3.4 kb (1.0 kb)	HL1032a
Human Eye cDNA	λgt10	Male/female eye pool.	1.1×10^6	0.4–3.2 kb (0.7 kb)	HL1047a
Human Eye cDNA	λgt11	Male/female eye pool.	1.5×10^6	0.4–2.8 kb (0.7 kb)	HL1047b
Human Fallopian Tube 5′-STRETCH cDNA	λ gt10	Adult fallopian tube, mid-section.	1.3×10^6	0.6 to>4.0 kb (1.5 kb)	HL1060a
Human Fallopian Tube 5′-STRETCH cDNA	λ gt11	Adult fallopian tube, mid-section.	1.2×10^6	0.6 to>4.0 kb (1.5 kb)	HL1060b
Human Fibroblast, Lung cDNA	λgt11	Lung fibroblast cell line, IMR-90. ATCC# CCL186.	2.2×10^6	0.5–3.0 kb (1.3 kb)	HL1011b
Human Fibroblast, Skin cDNA	λgt10	Cultured primary fibroblasts from a young male.	1.4×10^6	0.5–3.6 kb (1.0 kb)	HL1052a
Human Fibroblast, Skin cDNA	λgt11	Cultured primary fibroblasts from a young male.	1.1×10^6	0.5–3.5 kb (1.1 kb)	HL1052b

CLONTECH Libraries

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Fibrosarcoma cDNA	λgt11	HT-1080 tumor cells. Rasheed *et al., Cancer* 33: 1027 (1974). ATCC# CCL121.	1.6×10^6	1.0–3.4 kb (1.3 kb)	HL1048b
Human Genomic	EMBL3/ SP6/T7	Human Placenta DNA. *Sau*3A partial.	2.5×10^6	8–21 kb (15 kb)	HL1067j
Human Glioma cDNA	λgt11	HS 683 from explant cultures of a glioma taken from the left temporal lobe. ATCC # HTB 138.	1.1×10^6	0.4–3.1 kb (0.9 kb)	HL1049b
Human Hairy Cell Leukemia (Mo-B) cDNA	λgt10	Epstein-Barr virus-transformed B lymphoblast cell line. ATCC# CCL245.	1.4×10^6	0.4–3.2 kb (1.0 kb)	HL1043a
Human Heart cDNA	λgt11	Adult male heart, including some aorta region. Poly(A) RNA was slightly degraded as visualized on alkaline agarose gel.	1.27×10^6	0.4–3.4 kb (0.9 kb)	HL1038b
Human HeLa Cell cDNA	λgt11	HeLa-derived D98-AH2 cells, HPRT- in phenotype. W.S. Szybalski *et al., Natl. Cancer Institute Monograph*, 7: 75 (1962).	1.3×10^6	0.48–3.1 kb (0.86 kb)	HL1022b
Human Hepatoma cDNA	λgt11	Hepatoma cells, G2.	1.3×10^6	0.5–3.7 kb (0.96 kb)	HL1015b
Human HUT-78 T-Cell 5′-STRETCH, cDNA, PMA-activated	λgt11	Human T-cell line (*J. Exp. Med.* 154:1403(1981).) Positive for IL-2. ATCC # TIB 161.	1.5×10^6	1.0 to>4.0 kb (1.6 kb)	HL1068b
Human Keratinocyte cDNA	λgt11	Keratinocytes from adult epidermis.	1.7×10^6	0.5–3.8 kb (1.1 kb)	HL1045b
Human Kidney cDNA	λgt10	From a juvenile male whose kidney had been perfused for 24 hours prior to poly(A)$^+$ RNA isolation.	1.7×10^6	0.5–3.2 kb (0.9 kb)	HL1033a
Human Kidney cDNA	λgt11	See above.	1.4×10^6	0.4–3.1 kb (0.9 kb)	HL1033b
Human Kidney, Fetal, cDNA, 5′-STRETCH	λgt10	Fetal kidney tissues from a mixture of 20 week and 24 week old fetuses.	1.7×10^6	0.8 to>4.0 kb (1.6 kb)	HL1071a

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Kidney, Fetal, cDNA, 5′-STRETCH	λgt11	Fetal kidney tissues from a mixture of 20 week and 24 week old fetuses.	1.7 X 10^6	0.8 to>4.0 kb (1.6 kb)	HL1071b
Human Leukocyte Genomic	EMBL3	Leukocyte genomic DNA, male.	3.8 X 10^5	15kb	HL1036d
[illegible]n Leukocyte, Peripheral Blood, cDNA	λgt10	Peripheral blood leukocytes from adult female with Acute Promyelocytic Leukemia, HL60. S.J.Collins *et al.*, *Nature*, 270: 347 (1977). ATCC# CCL240.	7.2 X 10^5	0.69–3.5kb (0.88 kb)	HL1020a

[illegible]re 3. Human Peripheral Blood Leukocyte cDNA [illegible]ary in λgt11. Cat. No. HL1062b. Seven randomly selected clear plaques were subjected to analysis using two λgt11 GeneAmp™ PCR†† PRI-MATE™ Amplimers (Cat No. 5412-1).



Human Leukocyte, Peripheral Blood, cDNA	λgt11	See above.	8.4 X 10^5	0.77–3.6kb (1.1 kb)	HL1020b
[illegible]n Leukocyte, [illegible]al Blood, cDNA, 5′-STRETCH	λgt10	Adult male peripheral blood leukocytes.	1.7 X 10^6	1.2 to>4.0 kb (1.6 kb)	HL1062a
Human Leukocyte, Peripheral Blood, cDNA, 5′-STRETCH	λgt11	Adult male peripheral blood leukocytes.	1.8 X 10^6	0.8 to>4.0 kb (1.6 kb)	HL1062b
Human Liver cDNA	λgt10	Normal adult liver, female.	1.5 X 10^5	0.17–2.4 kb (0.8 kb)	HL1001a
[illegible] Liver cDNA	λgt11	Normal adult liver, female.	7.5 X 10^5	0.2–3.0 kb (1.1 kb)	HL1001b
Human Liver, Fetal, cDNA	λgt11	Fetal liver, 1st trimester, male.	2.3 X 10^5	0.[illegible]–2.3kb (1.[illegible] kb)	HL1035b

CLONTECH Libraries

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Liver, Fetal, cDNA, 5′-STRETCH	λgt10	Liver tissue from 22 week old fetus. Mother's blood type: O+.	1.7×10^6	1.2 to>4.0 kb (1.7 kb)	HL1064a
Human Liver, Fetal, 5′-STRETCH, cDNA	λgt11	Liver tissue from 22 week old fetus. Mother's Blood type: O+.	1.5×10^6	1.2 to>4.0 kb (1.6 kb)	HL1064b
Human Lung cDNA	λgt11	Normal adult lung tissue, excised during surgery.	8.2×10^5	0.25–3.1 kb (1.2 kb)	HL1004b
Human Lung, 5′-STRETCH cDNA	λgt11	Adult lung tissue, including trachea and bronchioles.	1.4×10^6	0.9 to>4.0 kb (1.5 kb)	HL1066b
Human Lung, Fetal, cDNA, 5′-STRETCH	λgt10	Fetal lung tissues from a mixture of 19 week and 21 week old fetuses.	1.4×10^6	0.6 to>4.0 kb (1.5 kb)	HL1072a
Human Lung, Fetal, cDNA, 5′-STRETCH	λgt11	Fetal lung tissues from a mixture of 19 week and 21 week old fetuses.	1.3×10^6	0.8 to>4.0 kb (1.6 kb)	HL1072b
Human Lung Fibroblast cDNA	λgt11	Lung fibroblast cell line, IMR-90. ATCC# CCL186.	2.2×10^6	0.5–3.0 kb (1.3 kb)	HL1011b
Human Lung Small Cell Carcinoma (NCI-H69) cDNA	λgt10	Lung Small Cell Carcinoma cell culture, NCI-H69.	1.4×10^5	0.6–3.6 kb (0.93 kb)	HL1012a
Human Lung Small Cell Carcinoma (NCI-H69) cDNA	λgt11	Lung Small Cell Carcinoma cell culture, NCI-H69.	7.7×10^5	0.4–2.8 kb (1.0 kb)	HL1012b
Human Lung Small Cell Carcinoma (NCI-H128) cDNA	λgt10	From NCI-H128 cell line derived from malignant pleural fluid of a 50 year old Black male. Gazder, *et al.*, *Cancer Research*, 40: 3502 (1980).	1.0×10^6	0.3–3.5 kb (0.85 kb)	HL1030a
Human Lung Small Cell Carcinoma (NCI-H128) cDNA	λgt11	From NCI-H128 cell line derived from malignant pleural fluid of a 60 year old Black male. Gazder, *et al.*, *Cancer Research*, 40: 3502 (1980).	1.3×10^6	0.3–3.7 kb (0.92 kb)	HL1030b
Human Lung, WI-38, cDNA	λgt11	WI-38 human diploid cell line, derived from normal Caucasian female lung. L. Hayflick, *Exp. Cell Res.*, 25: 585 (1961).	1.2×10^6	0.6–3.5 kb (1.1 kb)	HL1041b

Libr.	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Lymphocyte cDNA	λgt10	Near confluent Raji cells, B lymphocytes. Pulvertaft. *Lancet*, 1: 238 (1964). ATCC# CCL86.	1.5×10^6	0.25–3.2 kb (0.87 kb)	HL1002a
Human Mammary Gland		See Human Breast.			
[illegible]noma cDNA	λgt11	Near confluent melanoma A2058 cells.	7.1×10^5	0.6–4.0 kb (1.4 kb)	HL1023b
Human Monocyte, Peripheral Blood, cDNA	λgt11	90% human peripheral blood monocytes.	1.2×10^6	0.6–3.4 kb (1.1 kb)	HL1056b
Human Monocyte, Peripheral Blood, cDNA, LPS-activated	λgt10	90% human peripheral blood monocytes, LPS-activated.	1.34×10^6	0.4–3.5 kb (1.2 kb)	HL1050a
[illegible]nocyte, Blood, [illegible]NA LPS-activated	λgt11	90% human peripheral blood monocytes, LPS-activated.	1.3×10^6	0.5–3.7 kb (1.0 kb)	HL1050b
Human Monocyte, THP-1, cDNA	λgt10	THP-1 monocytes from 1 year old male with Acute Monocytic leukemia. *Int. J. Cancer*, 26: 171 (1980). *Cancer Research*, 42: 1530 (1982). ATCC# TIB202.	1.4×10^6	0.65–3.0 kb (0.97 kb)	HL1021a
[illegible] Monocyte, U-937, cDNA	λgt10	U-937 cultured cells actively growing prior to poly(A)$^+$ RNA isolation.	1.5×10^6	0.4–3.4 kb (0.95 kb)	HL1029a
Human Monocyte, U-937, cDNA	λgt11	See above.	1.2×10^6	0.3–3.7 kb (0.95 kb)	HL1029b
Human Monocyte, [illegible] PMA activated	λgt10	U-937 cells treated with 50ng/ml Phorbolmyristate acetate (PMA) for 3.5 days to achieve monocyte-like stage.	1.4×10^6	0.6–3.8 kb (1.1 kb)	HL1036a
Human Monocyte, U-937, PMA activated cDNA	λgt11	U-937 cells treated with 50ng/ml Phorbolmyristate acetate (PMA) for 3.5 days to achieve monocyte-like stage.	1.2×10^6	1.0–3.9 kb (1.3 kb)	HL1036b

CLONTECH Libraries

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Multiple Myeloma cDNA	λgt11	Bone marrow obtained from a female with Multiple Myeloma, IM9. *Ann.N.Y. Acad. Sci.*, 190: 221 (1972). *PNAS* 71: 84 (1974). *J. Biol. Chem.*, 249: 1661 (1974). ATCC# CCL159.	2.2×10^6	0.52–3.5 kb (1.1 kb)	HL1027b
Human Neuroblastoma cDNA	λgt10	Neuroblastoma cell line, Kelly.	1.05×10^5	0.4–3.4 kb (1.3 kb)	HL1007a
Human Osteosarcoma cDNA	λgt11	Osteosarcoma cell culture, MG-63. *Antimicrob. Ag. Chemother.*, 12: 11 (1977). ATCC# CRL1427	1.8×10^6	0.55–3.0 kb (0.97 kb)	HL1013b
Human Ovary cDNA	λgt10	Normal ovary from 31 year old caucasian.	1.8×10^6	0.6–4.0 kb (1.2 kb)	HL1098a
Human Ovary cDNA	λgt11	See above.	1.5×10^6	0.6–4.0 kb (1.4 kb)	HL1098b
Human Pancreas cDNA	λgt11	Adult male pancreatic tissue.	1.4×10^6	0.6–3.8 kb (1.2 kb)	HL1069b
Human Pancreas cDNA	SWAJ-2	Adult male pancreatic tissue.	1.7×10^6	0.6–3.5 kb (1.0 kb)	HL1069h
Human Pancreatic Carcinoma cDNA	λgt10	Pancreatic cell line HS 766T. Ref.: *J. Natl. Cancer Inst.* 56: 843 (1976). ATCC# HTB134.	1.1×10^5	0.5–3.4 kb (1.0 kb)	HL1057a
Human Pancreatic Carcinoma cDNA	λgt11	See above.	1.2×10^6	0.5–3.4 kb (1.0 kb)	HL1057b
Human Peripheral Blood Leukocyte cDNA		See Human Leukocyte, Peripheral Blood cDNA.			
Human Peripheral Blood Monocyte cDNA		See Human Monocyte, Peripheral Blood.			
Human Pituitary cDNA	λgt10	Tissue derived from a female with a gonadotropin-producing adenoma.	1.5×10^6	0.6–4.0 kb (1.2 kb)	HL1096a
Human Pituitary cDNA	λgt11	See above.	1.7×10^6	0.6–3.8 kb (1.2 kb)	HL1096b

Libr.	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Pituitary cDNA	λgt10	Tissue derived from a female with a growth hormone-producing adenoma.	1.4×10^6	0.5–3.8 kb (1.2 kb)	HL1097a
Human Pituitary cDNA	λgt11	See above.	1.3×10^6	0.6–4.0 kb (1.1 kb)	HL1097b
Placenta cDNA	λgt11	Placental tissue, 34 weeks old.	1.0×10^6	0.8–3.6 kb (1.8 kb)	HL1008b
Human Placenta cDNA, 5'-STRETCH	λgt11	Placental tissue, 30 weeks old.	2.4×10^6	1.2 to>4.0 kb (1.8 kb)	HL1075b
Human Prostate cDNA	λgt10	Normal 65 year old prostate tissues.	1.5×10^6	0.6–3.7 kb (1.2 kb)	HL1051a
Prostate cDNA	λgt11	Normal 65 year old prostate tissues.	1.62×10^6	0.5–3.6 kb (1.1 kb)	HL1051b
Human Retina cDNA	λgt10	Adult retina.	1.6×10^6	0.5–3.5 kb (1.1 kb)	HL1055a
Human Retina cDNA	λgt11	Adult retina.	1.4×10^6	0.5–3.4 kb (1.0 kb)	HL1055b
Human Skin Fibroblast	λgt10	Cultured primary fibroblasts from a young male.	1.4×10^6	0.5–3.6 kb (1.0 kb)	HL1052a
Human Skin Fibroblast cDNA	λgt11	Cultured primary fibroblasts from a young male.	1.1×10^6	0.5–3.5 kb (1.1 kb)	HL1052b
Human Spleen cDNA	λgt10	Normal 25 year old male spleen.	1.4×10^6	0.6–3.4 kb (1.0 kb)	HL1039a
Human Spleen cDNA	λgt11	Normal 25 year old male spleen.	1.3×10^6	0.6–3.3 kb (1.0 kb)	HL1039b
Human Spleen, CML, cDNA	λgt11	Spleen of 8 year old female with Chronic Myelogenous Leukemia.	1.4×10^6	0.5–3.8 kb (1.1 kb)	HL1040b
Human Stomach cDNA, 5'-STRETCH	λgt10	Edge of resectioned mid-portion of stomach from a 62 year old male with stomach carcinoma.	1.4×10^6	0.6 to>4.0 kb (1.4 kb)	HL1073a
Human Stomach cDNA, 5'-STRETCH	λgt11	See above.	1.7×10^6	0.6 to>4.0 kb (1.5 kb)	HL1073b
Human Submaxillary Gland cDNA	λgt11	Male gland excised during biopsy.	1.3×10^6	0.5–3.2 kb (1.0 kb)	HL1053b

CLONTECH Libraries

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human T-Cell cDNA	λgt10	T-Cell leukemic cell line. Jurkat. Schneider *et al., Int. J. Cancer*, 19:621 (1977). Yanagi *et al., PNAS*, 82: 3430 (1985).	1.1×10^6	0.58–3.8 kb (1.2 kb)	HL1016a
Human T-Cell cDNA	λgt11	See above.	1.1×10^6	0.58–3.8 kb (1.2 kb)	HL1016b
Human T-Cell, CEM cDNA, randomly-primed + oligo(dT)-primed	Zap†	CCRF-CEMT lymphoblastoid cell line (Foley *et al., Cancer* 18: 522 (1965).) Cell line is positive for CD4, CD5, T9 and T10, and negative for CD1, CD3, CD8, DR and TAC. Cell line is used for the propagation of HIV. ATCC # CCL 119.	1.6×10^6	1.2 to >4.0 kb (1.7 kb)	HL1063f
Human T-Cell, CEM cDNA, randomly-primed + oligo(dT)-primed	pBlue-script†	CCRF-CEMT lymphoblastoid cell line (Foley *et al., Cancer* 18: 522 (1965).) Cell line is positive for CD4, CD5, T9 and T10, and negative for CD1, CD3, CD8, DR and TAC. Cell line is used for the propagation of HIV. ATCC # CCL 119.	1.0×10^6	1.1–4.6 kb (2.3 kb)	HL1063g
Human T-Cell, HUT-78 **5′-STRETCH**, cDNA, PMA-activated	λgt11	Human T-cell line (*J. Exp. Med.* 154:1403 (1981).) Positive for IL-2. ATCC # TIB161.	1.5×10^6	1.0 to>4.0 kb (1.6 kb)	HL1068b
Human T-Cell, PEER **5′-STRETCH**, cDNA	λgt10	Human T-cell leukemic cell line, PEER.	1.8×10^6	1.0 to>4.0 kb (1.8 kb)	HL1078a
Human T-Cell, PHA Stimulated, cDNA	λgt10	Concentrated T-Cell population from peripheral blood of a healthy adult. PHA stimulated for 48 hours.	1.5×10^6	0.45–3.8 kb (0.95 kb)	HL1031a
Human T-Cell, PHA Stimulated, cDNA	λgt11	See above.	1.0×10^6	0.7–3.3 kb (1.4 kb)	HL1031b
Human Testis cDNA	λgt11	Normal testicle, excised during surgery from a healthy 50 year old. Not hormonally induced.	1.0×10^6	0.7–3.3 kb (1.2 kb)	HL1010b

Library	Vector	mRNA Source	# of Indep. Clones	Insert Size Range (Average)	Catalog Number
Human Thymocyte cDNA	λgt11	Normal thymus excised during surgery on a 3 year old Caucasian female.	1.7×10^6	0.45–3.4 kb (0.9 kb)	HL1026b
Human Thymus cDNA, 5'-STRETCH	λgt11	See above.	1.5×10^6	0.8 to>4.0 kb (1.6 kb)	HL1074b
Human Thymus cDNA, 5'-STRETCH	SWAJ-2	See above.	1.5×10^6	0.6 to>4.0 kb (1.5 kb)	HL1074h
Human Thyroid Carcinoma cDNA	λgt11	Adult thyroid carcinoma tissue, male.	6.3×10^5	0.65–2.7 kb (1.2 kb)	HL1009b
Human U251 cDNA, 5'-STRETCH	λgt10	Human U251 Astrocytoma.	1.5×10^6	1.0 to>4.0 kb (1.8 kb)	HL1077a
Human Wilms' Tumor cDNA	λgt11	Wilms' tumor cells derived from a 3 month old caucasian male. ATCC # CRL1441.	1.3×10^6	0.6–3.6 kb (1.2 kb)	HL1044b

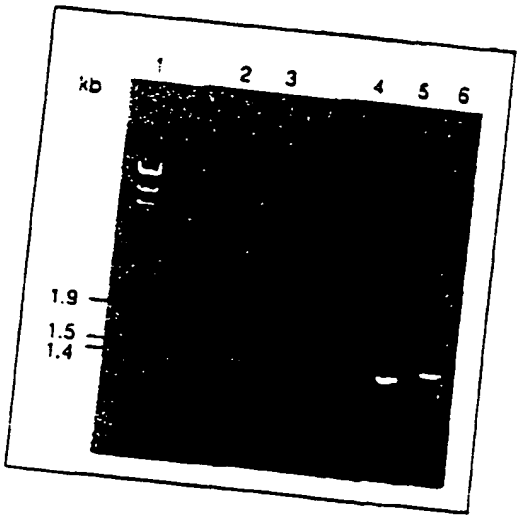


Figure 4. Mouse Brain cDNA library in λgt10, Cat. No. ML1024a. Four randomly selected clear plaques were subjected to analysis using two λgt10 GeneAmp™ PCR†† PRI-MATE™ Amplimers (Cat No. 5411-1).

Appendix H

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Molecular Cloning and Expression of the Human 55 kd Tumor Necrosis Factor Receptor

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Summary

Two distinct receptors for tumor necrosis factor (TNF) of 55 and 75 kd are expressed at low levels by various cells. The 55 kd TNF receptor was purified from HL60 cells, and partial amino acid sequences were determined. Short degenerate sense and antisense oligonucleotide primers encoding the N- and C-terminal ends of a peptide of 22 amino acid residues were used to amplify a 66 bp cDNA fragment from HL60 RNA by reverse transcriptase–polymerase chain reaction. The cDNA fragment as a probe identified several overlapping clones in a human placenta cDNA library. The open reading frame of the cDNA predicts a 455 amino acid TNF receptor protein with leader, extracellular, transmembrane, and intracellular domains. When expressed in COS-1 cells or in a baculovirus system, the cDNA conferred TNF binding properties comparable to the native receptor. Surprisingly, the 55 kd TNF receptor shows a high degree of sequence homology to the NGF receptor extracellular domain.

Introduction

TNF-α and -β (or cachectin and lymphotoxin, respectively; jointly referred to as tumor necrosis factor [TNF]) are two cytokines with close functional and evolutionary relation (Nedwin et al., 1985): they compete for the same cellular binding sites (Aggarwal et al., 1985) and their genes are located in close proximity within the major histocompatibility complex in mouse and in man (Spies et al., 1986; Mueller et al., 1987). TNF was originally characterized as a factor with anti-tumor activity (Old, 1985). However, the mechanism of tumor necrosis and regression remains poorly understood, even though TNF has been reported to exhibit direct cytotoxicity to many tumor cell lines in vitro (Matthews and Neale, 1987).

TNF has been found to possess a wide variety of biological activities. It exerts growth enhancing activity in fibroblasts, induces differentiation in human myeloid cell lines (for reviews, see Beutler and Cerami, 1987; Tracey et al., 1989), and has a crucial morphogenetic function in the induction and maintenance of granulomas in cell-mediated immunity (Kindler et al., 1989). Vascular endothelial cells are an important target for TNF. For example, TNF has been reported to induce expression of major histocompatibility HLA-A,B antigens or of adhesion-type molecules and to influence the morphology and proliferative activity of endothelial cell culture (Beutler and Cerami, 1987; Espevik et al., 1990). In other instances, TNF functions as mediator in immunologic and inflammatory responses. TNF concentrations in serum and cerebrospinal fluid correlate with the severity of inflammation in bacterial meningitis, with disease severity in malaria, or with mononuclear cell infiltration in acute phase graft-vs.-host disease (for reviews, see Beutler and Cerami, 1987; Tracey et al., 1989). TNF is one of the principal mediators of endotoxin in septic shock (Beutler et al., 1985; Rothstein and Schreiber, 1988; Michie et al., 1988).

Studies of intracellular signal transduction pathways revealed that TNF induces proteins that bind to κB-like enhancer elements and thus takes part in the control of NF-κB-inducible genes (Osborn et al., 1989; Lowenthal et al., 1989; Lenardo and Baltimore, 1989). The anti-viral activity of TNF at least in part is mediated by the interaction of NF-κB with a virus-inducible element in the β-interferon gene (Visvanathan and Goodbourn, 1989; Goldfeld and Maniatis, 1989). By an analogous mechanism, TNF appears to activate human immunodeficiency virus type I (Folks et al., 1989; Duh et al., 1989).

TNF-α in the crystal is packed in the form of a trimer of 17 kd monomeric units, and it is assumed that the trimer is the biologically active species (Hakoshima and Tomita, 1988; Eck et al., 1988; Jones et al., 1989). It cannot be excluded that a potential trivalency of the ligand, depending upon the surface density and lateral motility of receptors on various target cells, differentially affects receptor microclustering and thus has profound functional significance. In addition to the secreted 17 kd form, a 26 kd membrane-bound form of TNF is expressed in monocytes (Kriegler et al., 1988). It may function in intercellular contact or in paracrine fashion after posttranslational cleavage, resulting in the secretion of the 17 kd form.

The biological response to TNF is mediated by specific cell surface receptors. Several groups have reported studies on TNF receptors (Aggarwal et al., 1985; Creasy et al., 1987; Hirano et al., 1989; Hohmann et al., 1989; Kull et al., 1985; Niitsu et al., 1988; Smith and Baglioni, 1989; Tsujimoto et al., 1985). In TNF binding studies a single class of cell surface binding sites with a K_d in the pM range was identified on practically all of the cells analyzed. By cross-linking ^{125}I-TNF to the cell surface and SDS–PAGE, however, two or even more bands specifically binding TNF were detected (Kull et al., 1985; Creasy et al., 1987; Stauber et al., 1988; Hohmann et al., 1989; Smith and Baglioni, 1989). Furthermore, soluble TNF

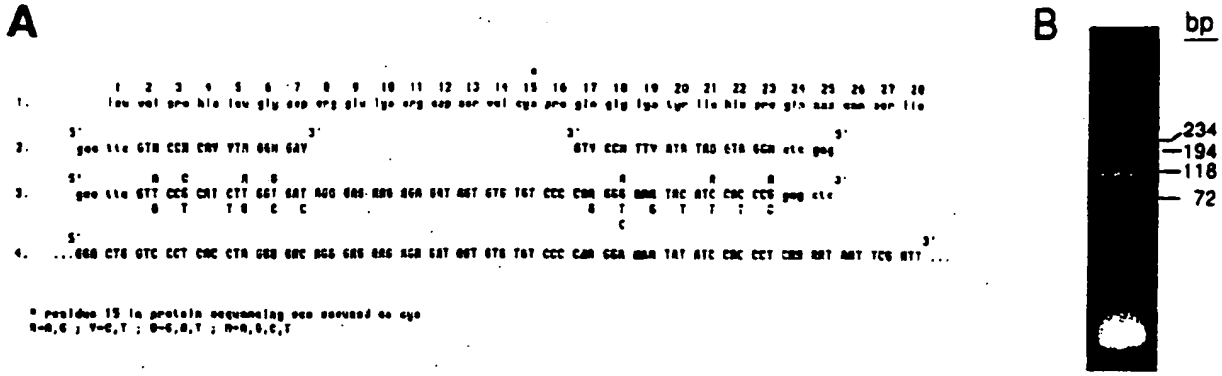

Figure 1. Partial Amino Acid Sequence of the 55 kd TNF Receptor and Gel Electrophoretic Analysis of the PCR Products

(A) Line 1: Sequence of the first 28 amino acids of the purified 55 kd TNF receptor. Residue 15 could not be asigned by protein sequencing but was assumed to be cysteine. Line 2: Location and sequences of the sense and antisense primer mixtures. The primers were used for first-strand cDNA synthesis and PCR. Line 3: Nucleotide sequences of ten individual cDNAs cloned in pUC19 from the 78 bp band (see B). Line 4: Nucleotide sequence of the 55 kd TNF receptor cDNA in the region of the 28 N-terminal amino acids.

(B) The product of the PCR amplification, starting from first-strand HL60 cDNA and using the sense and antisense primers defined in (A), line 2, was electrophoresed through a 12% nondenaturing polyacrylamide gel and stained with ethidium bromide. The DNA of the 78 bp lower band was cloned in pUC19, and the nucleotide sequence of ten clones was determined (see A, line 3).

binding and inhibiting protein(s) of about 30 kd in human serum and urine were discovered (Seckinger et al., 1989; Olsson et al., 1989; Engelmann et al., 1989). Recently, another molecule, called Fas antigen, was described that does not bind TNF by itself but appears to be associated with TNF receptors and may play a role in signal transduction (Yonehara et al., 1989). In view of these complexities it is obvious that the various molecules involved need to be analyzed, preferably in cloning and transfection studies, to gain insight into their functional significance in the TNF/TNF receptor system.

In our previous work we have identified two cell surface molecules of 55 and 75 kd apparent molecular mass that specifically bind TNF-α and -β with high affinity (Brockhaus et al., 1990; Hohmann et al., 1989). Specific monoclonal antibodies were raised to the purified molecules and were found to react exclusively with either the 55 or 75 kd molecule. The 55 kd molecule was found to be a functional TNF receptor, because anti-55 kd TNF receptor monoclonal antibodies elicited agonistic biological responses (Espevik et al., 1990). Here we report the molecular cloning and expression of the 55 kd TNF receptor and its relationship to the NGF receptor.

Results

Isolation of cDNAs Encoding the 55 kd TNF Receptor

The important steps in the isolation of 55 kd TNF receptor cDNA clones from a human placenta λgt11 cDNA library are outlined in Figure 1. The 55 kd TNF receptor protein was purified by combined ligand- and immunoaffinity chromatography and reverse-phase HPLC from a HL60 cell lysate (Loetscher et al., unpublished data). The sequence of the first 28 N-terminal amino acids (and of internal peptides; see below) was determined by protein sequencing as shown in Figure 1A. Fully degenerate primer oligonucleotides were synthesized that encode amino acids 17–23 (antisense primer) and 2–7 (sense primer) according to Figure 1A. Hexanucleotides containing the EcoRI and SacI endonuclease recognition sequences, respectively, were added to the 5′ ends of the primer oligonucleotides. Total RNA was purified from HL60 cells, and first-strand cDNA was generated with reverse transcriptase, using the antisense oligonucleotide mixture as primer. Finally, the cDNA fragment encoding amino acid residues 2–23 was amplified in a polymerase chain reaction (PCR) using the combined sense and antisense primers and the first-strand HL60 cDNA.

The PCR product was analyzed on a native polyacrylamide gel and was shown to yield two sharp bands with the lower band running at the expected size of 78 bp (Figure 1B). This latter fragment was cloned into pUC19 by the attached restriction sites, and ten clones were sequenced. The sequence of the region between the two primer oligonucleotides was found to be invariant and to encode the correct amino acid sequence. In contrast, quite a number of different primers were used in the PCR (Figure 1A). The 78 bp cDNA fragment was used as a probe, and it led to the isolation of one clone containing the complete open reading frame and of four partial clones from the human placenta cDNA library.

The Predicted 55 kd TNF Receptor Is a Transmembrane Protein

The sequence of the 55 kd TNF receptor cDNA is shown in Figure 2A. The predicted amino acid sequence reveals a number of interesting features. The complete sequence consists of 455 amino acid residues with typical hydrophobic putative leader and transmembrane sequences as predicted by hydrophilicity analysis (Devereux et al., 1984). The single 21 amino acid transmembrane region separates an N-terminal, presumably extracellularly located domain of 182 amino acids including 24 cysteines, three potential N-linked glycosylation sites, and a C-terminal domain of 223 residues

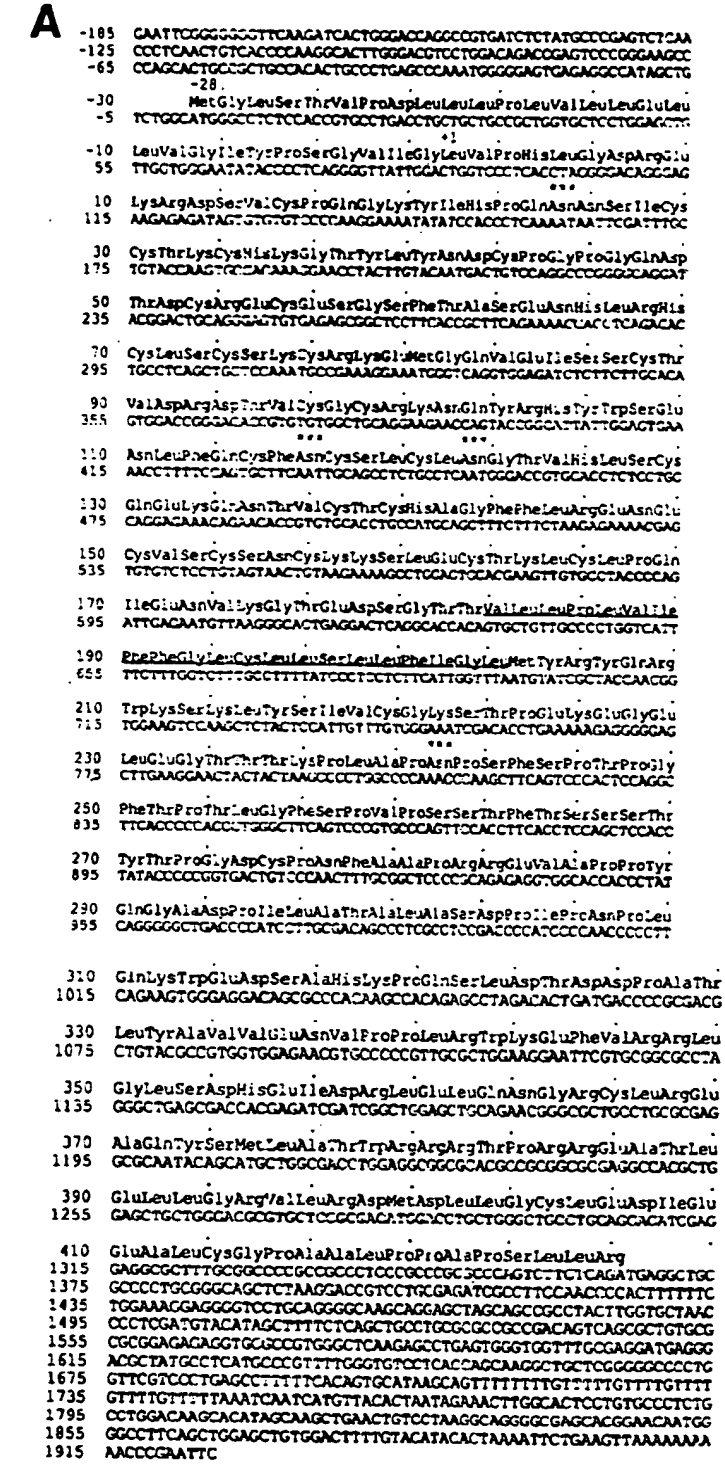

Figure 2. The 55 kd TNF Receptor Nucleotide and Predicted Amino Acid Sequences, and Schematic Representation of the 55 kd TNF Receptor cDNA Clone

(A) Amino acid numbering starts at the amino terminus Leu(+1); nucleotide numbering starts at the initiation codon. Amino acids 1–28 and 223–235 were also identified by sequencing the purified receptor protein. Residues 205–209 are in agreement with the receptor protein purified from human placenta (Loetscher *et al.*, unpublished data). The putative transmembrane region is underlined. Potential N-linked glycosylation sites are indicated by asterisks. For the cysteine residue pattern, see Figure 6.

(B) Hybridization probes A (SmaI–SmaI endonuclease-cut cDNA fragment), B (HindIII–EcoRI endonuclease-cut cDNA fragment), and C (EcoRI–EcoRI endonuclease-cut cDNA fragment) are indicated. The coding region of the cDNA is boxed and the putative leader (L), extracellular (EC), transmembrane (TM), intracellular (IC), and nontranslated (5′-nt, 3′-nt) regions are indicated.

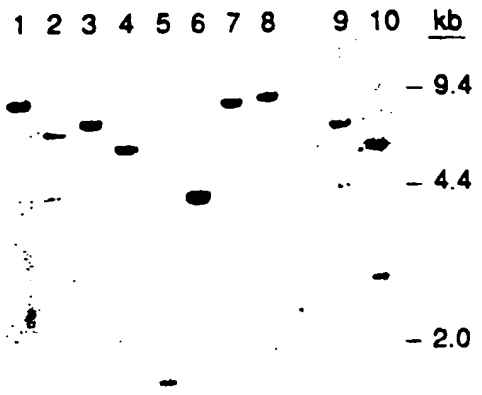

Figure 3 Genomic Southern Blot Analysis

Genomic DNA purified from HL60 cells was digested with restriction endonucleases (lane 1, EcoRI; lane 2, HindIII; lane 3, BamHI; lane 4, BamHI + EcoRI; lane 5, BamHI + HindIII; lane 6, SspI; lane 7, StuI; lane 8, ApaI; lane 9, ScaI + HindIII; lane 10, StuI + HindIII), electrophoresed through an agarose gel, and transferred to a nylon filter membrane. The filter was hybridized with probe B defined in Figure 2B as described in Experimental Procedures.

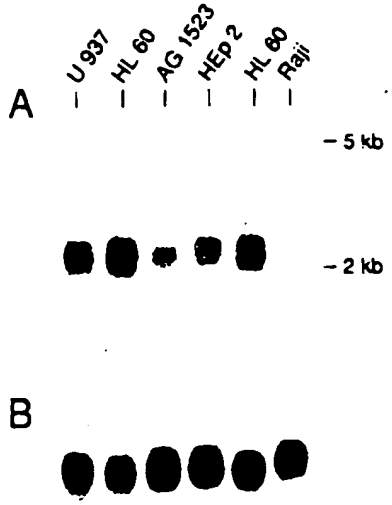

Figure 4. Northern Blot Analysis of Total RNA Purified from Various Cell Lines

(A) RNA (12 μg per lane) was electrophoresed through a formaldehyde-containing agarose gel and transferred to a nylon filter membrane. The filter was hybridized to probe B defined in Figure 2B.

(B) The same filter used in (A) was rehybridized after stripping to an actin probe as described in Experimental Procedures.

The reading frame in the C-terminal domain was confirmed by the sequence of an internal tryptic peptide fragment Ser(222)-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys. An independent confirmation was provided by the sequence of a cyanogen bromide fragment of the 55 kd TNF receptor purified from human placenta starting with Tyr(205)-Arg-Tyr-Gln-Arg (Loetscher et al., unpublished data). Interestingly, the soluble TNF inhibitory protein from human serum and urine described by several other investigators (Seckinger et al., 1989; Olsson et al., 1989; Engelmann et al., 1989) appears to be a soluble form of the 55 kd TNF receptor, because the 16–20 N-terminal amino acids described for the inhibitor are identical to a sequence of the 55 kd TNF receptor starting at aspartic acid(12).

A 29 amino acid leader sequence precedes the leucine identified by protein sequencing as amino terminus of the isolated mature protein leucine(+1); this predicts a signal peptide cleavage site that obeys the (−3, −1) rule (von Heijne, 1986), and thus leucine(+1) may represent the true N-terminus of the mature protein molecule. A more thorough analysis of a weight matrix of eukaryotic signal sequences, taking into account residues −13 to +1, reveals, however, a potentially more likely cleavage site at the Gly(−9)–Ile(−8) peptide bond (von Heijne, 1986).

An unusual feature of the 55 kd TNF receptor sequence is the very high cysteine content in the extracellular domain (24 cysteines in a total of 182 residues). This explains why the ligand binding to the 55 kd TNF receptor is highly sensitive to reducing agents (Loetscher et al., unpublished data) and supports the hypothesis that the N-terminal sequence is the ligand binding and therefore extracellular domain. The high proline content of the receptor (34 residues) is another unusual feature expected from the results of amino acid composition analysis.

The 55 kd TNF Receptor Gene Is Unique

Genomic Southern blots of HL60 DNA were hybridized with the cDNA probes A, B, and C defined in Figure 2B, which are located up- and downstream of the internal EcoRI site, respectively (see Figure 3 for hybridization with probe B; data for probes A and C are not shown). The two probes B and C identified two different bands in the EcoRI digest, whereas both probes identified the same bands in the BamHI, HindIII, SspI, StuI, and ApaI digests. Probe B hybridized with one additional band in HindIII digests, which is readily explained by a HindIII restriction site in an intron located between the HindIII and EcoRI sites of the cDNA clone. Similarly, probe C hybridized with one additional band in ApaI digests as a consequence of the ApaI site in the 3′ nontranslated region of the cDNA clone. Probes A and B hybridized to the same bands in the EcoRI, BamHI, and ApaI digests. In HindIII digests, probe A identified a band of the same size as one that hybridized to probe B, but not to probe C. In SspI and StuI digests, probe A hybridized with bands that were different from those identified by both probes B and C. The structure of the 55 kd TNF receptor gene must be investigated in a more detailed analysis of genomic DNA; however, it appears from the present data that a single gene with at least three exons codes for the 55 kd TNF receptor.

The 55 kd TNF Receptor Is Expressed in HL60, U937, AG1523, and HEp2 Cells, but Not in Raji Cells

The pattern of 55 kd TNF receptor expression in HL60, U937, HEp2, AG1523, and Raji cells was studied by Northern blot analysis (Figure 4). A single mRNA species was

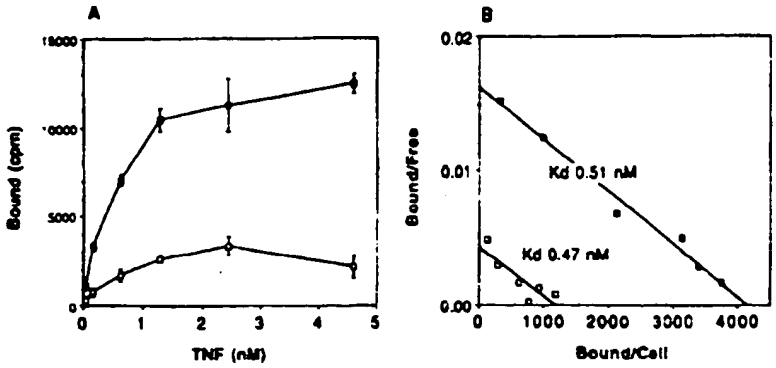

Figure 5. TNF Binding and Scatchard Analysis of COS-1 Cell Transfectants

(A) TNF binding. Monolayers of COS-1 cells transfected with the 1.3 kb EcoRI–EcoRI 55 kd TNF receptor cDNA as described in Experimental Procedures and nontransfected COS-1 cells in parallel were incubated with various concentrations of ^{125}I-TNF-α for 2 hr at 4°C and assayed for binding. Black squares, transfected COS cells; open squares, nontransfected control.

(B) Scatchard analysis of the binding data.

identified in HL60, U937, HEp2, and Ag1523 cells, whereas Raji cells appeared to be practically devoid of 55 kd TNF receptor mRNA. These findings are in agreement with results of Western blot and flow cytometry studies using an anti–55 kd TNF receptor monoclonal antibody where HL60, U937, and HEp2 cells were clearly receptor positive, whereas no antigen was detected in Raji cells (Brockhaus et al., 1990). All three cDNA fragments (probes A, B, and C defined in Figure 2B) hybridized to the same band on the Northern blot. No evidence for the existence of a second mRNA species was obtained with probe A, which is specific for both the 55 kd TNF receptor and the TNF inhibitor sequences (Seckinger et al., 1989; Olsson et al., 1989; Engelmann et al., 1989).

Transfection and Expression of the 55 kd TNF Receptor cDNA Confers TNF Binding Activity

To establish that the 55 kd TNF receptor cDNA carries the complete minimum information required to confer specific TNF binding to a recipient cell, two independent expression systems were investigated. First, the 1.3 kb EcoRI–EcoRI fragment (Figure 2B) was cloned in a modified pXF3/ori vector, which contains the human cytomegalovirus immediate-early promoter and SV40 ori (Cullen, 1986). This construct was transfected into COS-1 cells (Gluzman, 1981) using lipofectin, and transient expression was measured (Felgner et al., 1987). Specific ^{125}I-TNF binding to the surface of the transfected cells was analyzed after 3 days in culture in the absence and presence of a 500-fold excess of cold TNF. The binding data and Scatchard analysis are presented in Figure 5. The K_d of about 0.5 nM determined with the COS cell transfectants is comparable to the K_d of the native 55 kd TNF receptor as it is expressed almost exclusively on HEp2 cells (Hohmann et al., 1989). The endogenous TNF binding sites of COS cells have affinities comparable to those of the human 55 kd TNF receptor type. Furthermore, it was found that a monoclonal antibody, htr-9 (Brockhaus et al., 1990), directed to the human 55 kd TNF receptor extracellular domain inhibits TNF binding to the endogenous COS cell receptor almost completely. We assume, therefore, that the predominant endogenous COS cell TNF receptor is the homolog of the human 55 kd TNF receptor. Immunofluorescence studies using the anti–55 kd TNF receptor antibody (see above) show that the relatively low degree of expression of the 1.3 kb EcoRI–EcoRI fragment receptor construct is due to the fact that the ^{125}I-TNF binding measured in the assay (Figure 5) results from a small number of strongly positive transfectants, whereas the vast majority of cells is practically not stained.

Second, the 55 kd TNF receptor expression was investigated in a baculovirus expression system. The 1.3 kb EcoRI–EcoRI fragment was cloned in a modified pVL941 plasmid under the control of the polyhedrin promoter and introduced into the AcNP virus by homologous recombination (Luckow and Summers, 1988). When Sf9 cells were infected with the virus construct a highly significant and specific cell surface TNF binding was observed (see Table 1).

The 55 kd TNF Receptor and the NGF Receptor Have Highly Similar Extracellular Domains

A highly significant sequence similarity of the 55 kd TNF receptor with the nerve growth factor (NGF) receptor (Johnson et al., 1986; Radeke et al., 1987) was found. The alignment of both receptor sequences scored 13.6 standard deviations above the random score with the Mutation Data Matrix (Dayhoff et al., 1979). The similarity is most striking in the extracellular domains, where a block of 46 amino acids containing six regularly spaced cysteines is repeated four times in the two receptors (Figure 6). With suitable gapping, these domains containing 24 cysteines can be aligned, yielding 58 identities out of 169 possible matches (34% identity).

Table 1. TNF Binding with Sf9 Cells Infected with the 55 kd TNF Receptor cDNA AcNP Virus Construct

Cells	Specific ^{125}I-TNF Bound per 1 × 10^6 Cells[a]
Noninfected Cells (Control)	60 cpm
Infected Cells[b]	1600 ± 330 cpm

[a] Virus construct, infection, and binding assay as described in Experimental Procedures.
[b] Average and SD of four experiments.

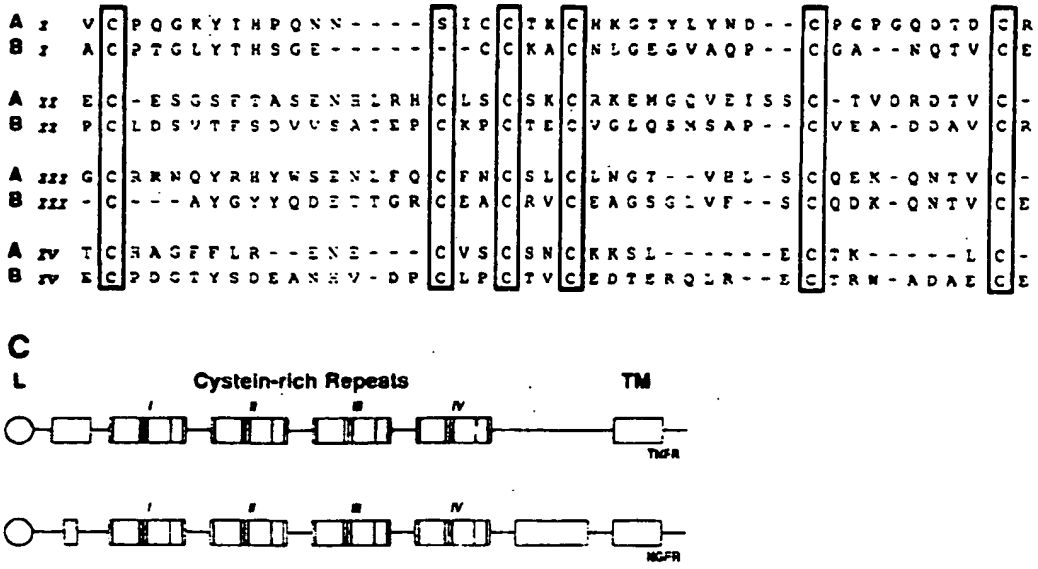

Figure 6. Homology between the 55 kd TNF Receptor and the NGF Receptor

(A) and (B) Four homologous cysteine-rich repeating elements in the extracellular domains of the 55 kd TNF receptor and the NGF receptor are shown (Johnson et al., 1986; Radeke et al., 1987). (A) 55 kd TNF receptor (I: residues 14–53; II: 54–96; III: 97–137; IV: 138–166). (B) Human NGF receptor (I: residues 4–36; II: 39–79; III: 81–118; IV: 121–160) (Johnson et al., 1986).

(C) Schematic comparison of the 55 kd TNF receptor and NGF receptor extracellular domain sequences.

Discussion

Our strategy for the molecular cloning of the 55 kd TNF receptor relied to a large extent on PCR technology (Saiki et al., 1985). Despite its low abundancy, sufficient protein was purified to allow for the determination of 28 N-terminal and 15 internal amino acid residues. In preliminary experiments, the more conventional cloning approach using relatively short, fully degenerate or longer best-guess oligonucleotides as probes to screen cDNA libraries had proven technically difficult. An improved hybridization probe, therefore, was generated by PCR with degenerate primers and employed to identify the correct cDNA clone. A similar approach was used previously for the cloning of another, significantly more abundant gene (Lee et al., 1988).

The 55 kd TNF receptor has the typical structure of a membrane-spanning protein with a single transmembrane region separating intra- and extracellular domains. The calculated molecular mass of the mature protein is 47.5 kd. Because the purified receptor migrates with an apparent molecular mass of 55 kd on SDS–PAGE, post-translational modifications, most likely glycosylations, must account for the difference. Deglycosylation by N-glycanase treatment has been found to reduce the apparent molecular mass of the mature receptor by 5–10 kd (Hohmann et al., 1989; Loetscher et al., unpublished data), and therefore one or the other of the N-linked glycosylation sites appears to be utilized.

Recently, several lymphokine receptors, such as the erythropoietin receptor or the β chain of the IL-2, IL-4, and IL-6 receptors, have been shown to share extensive sequence homologies and to form a new interleukin receptor gene family (D'Andrea et al., 1989; Mosley et al., 1989; Yamasaki et al., 1988). A distinctive element of this family is the Trp-Ser-X-Trp-Ser sequence motif found in the extracellular domain adjacent to the transmembrane region. The 55 kd TNF receptor lacks this motif and does not belong to this receptor family; rather, it is homologous in sequence to the NGF receptor, the sequence similarity being strongest in the extracellular domain, and belongs to the NGF/EGF/LDL receptor family.

In view of the potent bioactivity of TNF, a TNF inhibitor could have an important physiological role. The significance of the recently discovered TNF inhibitor peptide of human serum and urine (Olsson et al., 1989; Seckinger et al., 1989; Engelmann et al., 1989) is not yet understood, but it might function as a TNF sink. Our finding that the first 20 amino acids of the inhibitor match the 55 kd TNF receptor sequence starting at residue 12 of the mature receptor leaves little doubt that the inhibitor is a soluble fragment of the receptor molecule, probably containing most of the extracellular domain. Soluble forms of other lymphokine receptors, such as the IL-2 receptor α chain, were reported previously (Rubin et al., 1985), and a specific mRNA encoding a soluble IL-4 receptor has been identified (Mosley et al., 1989). The genomic Southern blot analysis of the 55 kd TNF receptor provides no evidence for the existence of a distinct second gene that might encode the inhibitor. Furthermore, no evidence for the existence of a shorter mRNA species potentially encoding the inhibitor and created, e.g., by differential splicing, was found by Northern blot analysis of various cell lines. A specific TNF inhibitor mRNA, however, might be the result of a tissue-specific splicing event and thus need not be detected with the cell lines used in the present study. It is therefore possible that the TNF inhibitor is encoded by a tissue-specific differentially spliced transcript.

It seems more likely, however, that it is created by proteolytic processing of the receptor molecule. With regard to the latter possibility, the finding of two amino termini, i.e., leucine(+1) in the receptor and aspartic acid(12) in the inhibitor (Seckinger et al., 1989; Olsson et al., 1989; Engelmann et al., 1989), and of a potential third signal peptide cleavage site predicted by sequence comparison, isoleucine(−8), is intriguing. It cannot be excluded that a primary translation product undergoes extensive posttranslational processing, which leads to the mature receptor and inhibitor molecules.

Several cell surface receptors have been found to consist of two or more peptide chains; for example, the IL-2 receptor system comprises at least a β and an inducible α chain, which function individually or as a complex (Hatekeyama et al., 1989). The TNF receptor system is no exception in this context: at present it comprises the 55 kd and 75 kd TNF binding molecules and the so-called Fas antigen (Yonehara et al., 1989). The 55 kd molecule, molecularly characterized in this study, is able to bind TNF and, upon binding of a specific antibody, to elicit biological responses in sensitive cells (Espevik et al., 1990). The availability of the 55 kd TNF receptor cDNA will allow investigation of TNF function and signal transduction in a more precise way than previously possible; it will also allow assessment of the roles of the 75 kd TNF binding protein and of other, potentially associated chains.

Experimental Procedures

Cell Lines and Growth Conditions

The human cell lines HL60 (ATCC CCL 240), U937 (ATCC CRL 1593), HEp2 (ATCC CCL 23), and Raji (ATCC CCL 86) and the dermal fibroblast line AG-1523 (Camden Cell Depository) were grown in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse or inactivated fetal calf serum. COS-1 cells (ATCC CRL 1650) were maintained in DMEM with 10% inactivated FCS. Sf9 insect cells (ATCC CRL 1711) were cultivated as described (Luckow and Summers, 1988).

Reagents

Recombinant human TNF-α purified from Escherichia coli was a gift from Drs. W. Hunziker, E. Hochuli, and B. Wipf (Hoffmann-LaRoche LTD., Basel). TNF-α was radioiodinated with Na ^{125}I (IMS40 Amersham) and Iodo-Gen (Pierce) to 0.3–1.0 × 10^6 cpm/mg as described (Fraker and Speck, 1978). Synthetic oligonucleotides were purchased from commercial sources.

Purification and Sequencing of the 55 kd TNF Receptor Protein

The 55 kd TNF receptor protein was purified to apparent homogeneity from HL60 cells by combined ligand- and immunoaffinity chromatography followed by reverse-phase HPLC on a Pharmacia ProRPC 5/2 column (Loetscher et al., unpublished data). Specific TNF binding activity of the purified protein was demonstrated in a solid phase assay (ligand blot). Briefly, proteins were separated by nonreducing SDS–PAGE and then electrophoretically transferred to a PVDF membrane (Matsudaira, 1987; Towbin et al., 1979). The membrane was blocked with 1% defatted milk powder and incubated with 5 ng/ml ^{125}I-TNF-α in the presence and absence of 5.0 μg/ml unlabeled TNF-α. TNF binding was detected by autoradiography.

An aliquot of the HPLC-purified receptor protein was subjected to automated Edman degradation, and the first 28 amino acids were determined. Additional sequence information was obtained after reduction and S-carboxymethylation of the protein (Jones, 1986), followed by cyanogen bromide cleavage (Tarr, 1986) and subsequent trypsin digestion in 200 mM NH_4HCO_3 (pH 8.0) (1% estimated molar ratio trypsin, added twice at 16 hr intervals at 37°C). The resulting peptide fragments were separated by reverse-phase HPLC on a Brownlee Aquapore RP 300 column (100 × 2.1 mm) and subjected to N-terminal sequence analysis on an Applied Biosystems 475A protein sequencer with online PTH-amino acid analyzer (Hewick et al., 1981).

To analyze similarities with other known sequences, sequence library searches and alignments were performed using the combined GenBank, National Biomedical Research Foundation, European Molecular Biology Laboratory, Protein Research Foundation, and Swiss-Prot data bases; computer programs were obtained from the Genetics Computer Group, the National Biomedical Research Foundation, and Hoffmann-LaRoche (Dayhoff et al., 1979; Devereux et al., 1984; Pearson and Lipman, 1988).

Polymerase Chain Reaction

First-strand cDNA was synthesized with total RNA purified from HL60 cells and a cDNA synthesis kit (Amersham) according to the instructions of the manufacturer, except that the degenerate antisense oligonucleotide mixture (Figure 1A) was used as primer for the reverse transcriptase. First-strand HL60 cDNA in a mixture with the sense and antisense primer oligonucleotides (Figure 1A) was subjected to PCR using a Cetus GeneAmp kit and a Perkin-Elmer Thermocycler. The conditions were modified such that the primer concentrations were partially corrected for the level of their degeneracy. The PCR was run for 25 cycles (30 s at 94°C; 90 s at 55°C; 90 s at 72°C). An aliquot of the reaction was electrophoresed through a 12% nondenaturing polyacrylamide gel, bands were visualized by ethidium bromide staining and excised, and the DNA was recovered by electrophoretic transfer onto DEAE–cellulose paper as described (Maniatis et al., 1982). To determine the nucleotide sequences, the PCR-amplified cDNA fragments were cloned in pUC19 vector. To obtain a screening probe the 78 bp fragment was labeled by PCR using 10 mM [α-^{32}P]dCTP instead of 200 mM dCTP in the amplification reaction.

cDNA Library and Plaque Screening

A human placenta cDNA library ligated into λgt11 vector was purchased from Clontech (1.0 × 10^6 independent clones, 1.8 kb average insert size). The amplified library was plated, and duplicate plaque lift filters (GeneScreen Plus) were prepared according to standard protocols (Maniatis et al., 1982; Ausubel et al., 1989). The filters were hybridized to the denatured 78 bp cDNA probe (^{32}P-labeled by PCR) and washed as recommended by the manufacturer. Filters were exposed to X-Omat AR5 film with Cronex Li-Plus enhancer screen at −70°C overnight. Double positive clones were plaque purified, and the insert DNA was cloned in pUC and M13mp vectors according to standard protocols (Ausubel et al., 1989). DNA sequencing was performed with a Sequenase sequencing kit (US Biochemical).

Blot Transfer and Hybridization Protocols

For Northern blot analysis, total RNA purified from various cell lines was electrophoresed through 1.0% agarose gels containing formaldehyde as described (Maniatis et al., 1982). After electrophoresis gels were blotted by capillary transfer in 10× SSC to Zeta-Probe nylon membranes (BioRad). Southern blot analysis was performed by alkaline blotting DNA separated on agarose gels to Zeta-Probe nylon membranes as recommended by the manufacturer. cDNA probes were either labeled by random priming (DNA labeling kit; Boehringer Mannheim) or by PCR. Hybridization, washing, and stripping of the Zeta-Probe membranes was carried out in SDS buffer according to the instructions of the manufacturer. The final high stringency wash was in 40 mM NaH_2PO_4, 1 mM EDTA, 1% SDS at 65°C performed twice for 30 min.

Cell Transfection, Expression, and Cellular Binding Assays

For transient COS-1 cell transfections (Gluzman, 1981), the 1.3 kb EcoRI–EcoRI fragment of the 55 kd TNF receptor cDNA was cloned in a modified pXF3/ori vector (Cullen, 1986), which contains the human cytomegalovirus immediate-early promoter and SV40 ori, and the construct at 1.8 μg/ml DNA was introduced into COS-1 cells by lipofectin-induced transfection (Felgner et al., 1987). After 2–3 days in culture the cells were detached with EDTA (Gibco) and tested for ^{125}I-TNF-α binding. The cells were washed, resuspended at 2.8 × 10^6 cells/ml, and incubated with various concentrations of ^{125}I-TNF-α in the absence

and presence of a 500-fold excess of cold TNF-α for 2 hr at 4°C. The bound radioactivity was counted in a γ counter. Nonspecific binding was subtracted. Scatchard analysis was performed by the ligand algorithm (courtesy of P. J. Munson).

For the baculovirus expression system the 1.3 kb EcoRI–EcoRI fragment was cloned in a modified pVL941 plasmid under the control of the polyhedrin promoter and introduced into the AcNP virus by homologous recombination (Luckow and Summers, 1988). Sf9 insect cells (ATCC CRL 1711) were infected, and after 3 days in culture the specific cell surface TNF-α binding was measured. The Sf9 cells were washed from the culture dish with a Pasteur pipet, resuspended at 5×10^6 cells/ml in Sf9 medium containing 10 ng/ml ^{125}I-TNF-α in the absence and presence of 5 μg/ml unlabeled TNF-α, and incubated for 2 hr on ice. The cells were washed with Sf9 medium, and the bound radioactivity was counted in a γ counter.

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GenBank Accession Number

The accession number for the sequence reported in this paper is M33480.

Appendix I

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Molecular Cloning and Expression of a Receptor for Human Tumor Necrosis Factor

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Summary

A human tumor necrosis factor (TNF) binding protein from serum of cancer patients was purified to homogeneity and partially sequenced. Synthetic DNA probes based on amino acid sequence information were used to isolate cDNA clones encoding a receptor for TNF. The TNF receptor (TNF-R) is a 415 amino acid polypeptide with a single membrane-spanning region. The extracellular cysteine-rich domain of the TNF-R is homologous to the nerve growth factor receptor and the B cell activation protein Bp50. Human embryonic kidney cells transfected with a TNF-R expression vector specifically bind both ^{125}I-labeled and biotinylated TNF-α. Unlabeled TNF-α and TNF-β were equally effective at displacing the binding of labeled TNF-α to TNF-R expressing cells. Northern analysis indicates a single species of mRNA for the TNF-R in a variety of cell types. Therefore, the soluble TNF binding protein found in human serum is probably proteolytically derived from the TNF-R.

Introduction

Tumor necrosis factor–alpha (TNF-α) is a multipotent cytokine produced mainly by activated macrophages. TNF-α was originally identified as a tumoricidal protein effecting hemorrhagic necrosis of transplanted solid tumors in mice (Carswell et al., 1975) but has since been implicated in diverse biologic processes including inflammation and immunoregulation, antiviral defense, endotoxic shock, cachexia, angiogenesis, and mitogenesis (Goeddel et al., 1986; Beutler and Cerami, 1988; Old, 1988). The related cytokine lymphotoxin (TNF-β) is synthesized by activated lymphocytes and shares many of the biological activities of TNF-α (Goeddel et al., 1986).

The mechanisms through which the TNFs mediate their multiple activities are largely unknown, but like most polypeptide hormones, binding to specific cell surface receptors is an initial event. Stable trimers comprised of identical TNF-α polypeptides of 17,350 daltons bind to sites on a variety of cell types, with dissociation constants (K_d) ranging from 1.3×10^{-9} M to 7.1×10^{-11} M (Aggarwal et al., 1985; Kull et al., 1985; Tsujimoto et al., 1985; Baglioni et al., 1985; Watanabe et al., 1986; Tsujimoto and Vileck, 1987; Stauber et al., 1988; Hohmann et al., 1989; Ding et al., 1989). While most investigators report a single class of cell surface binding sites, others report the presence of both high ($K_d = 2.6 \times 10^{-13}$ M) and low ($K_d = 1.5 \times 10^{-10}$ M) affinity sites on the same cell (Imamura et al., 1987). TNF-β and TNF-α have been shown to compete for binding to the same receptor on the human cervical carcinoma cell line ME-180 (Aggarwal et al., 1985) and the histiocytic lymphoma cell line U-937 (Stauber and Aggarwal, 1989). Estimates of the size of the TNF receptor (TNF-R) determined by affinity labeling studies range from 54 to 175 kd (Creasey et al., 1987; Stauber et al., 1988; Hohmann et al., 1989; Smith and Baglioni, 1989). A recent report suggests the existence of two major receptor types for TNF-α: a myeloid cell type receptor with a K_d of 7.1×10^{-11} M and an epithelial cell type receptor with a K_d of 3.2×10^{-10} M. These two receptor types differ in size, glycosylation, and in their peptide maps (Hohmann et al., 1989).

In addition to the cell surface receptors for TNFs, several groups have identified soluble proteins in human urine (Peetre et al., 1988; Seckinger et al., 1988, 1989; Engelmann et al., 1989, 1990) and in the serum of human cancer patients (Gatanaga et al., 1990) capable of specifically binding TNFs. In one instance two immunologically distinct TNF binding proteins (TNF BPs) were isolated from human urine (Engelmann et al., 1990). Antibodies raised against those two proteins (TBP I and TBP II) had an inhibitory effect on the binding of TNF-α to its cell surface receptor, suggesting a structural similarity between the cell surface TNF-R and the soluble TBPs.

Soluble cytokine binding proteins in biological fluids have been shown in some cases to represent "shed" forms of cell surface cytokine receptors (Rubin et al., 1985; Novick et al., 1989; Zupan et al., 1989). To ascertain whether this was the case for the TNF-R, we purified a soluble TNF BP from human serum and isolated a corresponding cDNA by molecular cloning. This cDNA encodes a cell surface receptor for TNF that can presumably be processed to yield a soluble TNF BP.

Results

Purification and Characterization of TNF BP

A protein that inhibits the activity of both TNF-α and TNF-β has been detected in the serum of cancer patients but not healthy individuals (Gatanaga et al., 1990). This protein was purified by TNF-α affinity chromatography. Proteins eluted from the TNF-α affinity column were separated by reverse-phase HPLC, and several residues of N-terminal amino acid sequence were determined for each. Only one sequence was obtained, DSV(C/H)PQGKYIH, that did not correspond to a known serum protein. This protein, with an apparent M_r of 28,000, showed N-terminal sequence

son et al., 1989; Engelmann et al., 1989, 1990). To obtain the amino acid sequence of internal peptides, the HPLC-purified TNF BP was subject to proteolysis using lysine C in the presence of SDS and the resulting proteolytic fragments were separated. The sequences of the two major eluting peaks were determined: GTYLYNDCPGPGQDENE for PF I and EMGQVEISSCTVDNDTVCG for PF II.

cDNA Cloning and Characterization Reveal a Receptor Structure

Two synthetic DNA probes were synthesized based on the amino acid sequence of PF I and PF II, using human codon bias (Lathe, 1985). The two probes were used to screen cDNA libraries made from placental tissue and from the promyelocytic cell line HL-60. Several positive clones were obtained from both libraries. The DNA sequence was determined for four overlapping cDNA clones from the HL-60 library. The composite sequence contained a single long open reading frame. The sequence of a 2.1 kb cDNA from the placental library was also determined and found to overlap the combined sequences of the HL-60 clones. The placental cDNA clone contains all of the presumed coding region as well as some of the 5′ and 3′ untranslated regions. The composite nucleotide sequence of the cDNAs and the deduced amino acid sequence of the predicted protein is shown in Figure 1A. There are three nucleotide differences between the placental and HL-60 clones (A at nucleotide position 75 in the placental vs. G in the HL-60, G vs. A at position 219, and G vs. A at position 1342), none of which results in an amino acid change. The open reading frame defines a protein of 455 amino acids starting at nucleotide position 182 and terminating at nucleotide position 1545.

The encoded protein exhibits a predicted domain structure typical of a cell surface receptor: the hydropathy profile indicates a signal peptide at the beginning of the protein and a potential transmembrane domain in the middle that separates the presumed extra- and intracellular domains (Figure 1B). The 11 amino acids designated +1 through 11 match the N-terminal amino acids of the soluble TNF BP isolated from serum. Therefore, we assigned Asp+1 as the N-terminal residue, although it is not known whether the N-terminus of the cell surface form of this molecule is the same as the N-terminus of the soluble form that was sequenced. Residues −40 to −10 are largely hydrophobic and probably serve as a signal peptide. Although the precise cleavage point is not known, the Gly (−12)–Leu (−11) peptide bond is a possible site (von Heijne, 1986). The mature protein may result from further proteolytic processing at the basic Lys (−2)–Arg (−1) dipeptide. Residues +1 through 172 probably constitute a cysteine-rich extracellular domain, with 24 cysteine residues and 3 potential sites for N-glycosylation (Asn-X-Ser/Thr) at residues 14, 105, and 111. In addition to the identity to the N-terminal sequence of soluble TNF BP, this domain contains sequences corresponding to the lysine C–generated proteolytic fragments PF I (residues 24–41) and PF II (residues 67–86). The 23 amino acid hydrophobic region in the middle of the molecule, which is flanked on its amino-terminal side by Thr 171 and on its carboxy-terminal side by Arg 195, is characteristic of a transmembrane-spanning domain. The putative cytoplasmic domain would be comprised of the remaining 220 amino acids.

Cysteine Repeats in the Extracellular Domain of the TNF-R

In the presumed mature extracellular domain of the predicted TNF-R protein, 24 of the 171 total amino acids are cysteines and the spacing of the cysteine residues is periodic. In contrast, the remaining 265 residues contain only 6 cysteines. Dot matrix analysis of these regions using the ALIGN score and the Dayhoff matrix (Dayhoff et al., 1978, 1983) reveals significant diagonal patterns of homology, indicating internal homologies (Figure 2A). Inspection of the extracellular domain sequence reveals it can be roughly divided into four related subdomains (Figure 2B). This 4-fold symmetry may represent duplication events of an ancestral subdomain in the evolution of the TNF-R.

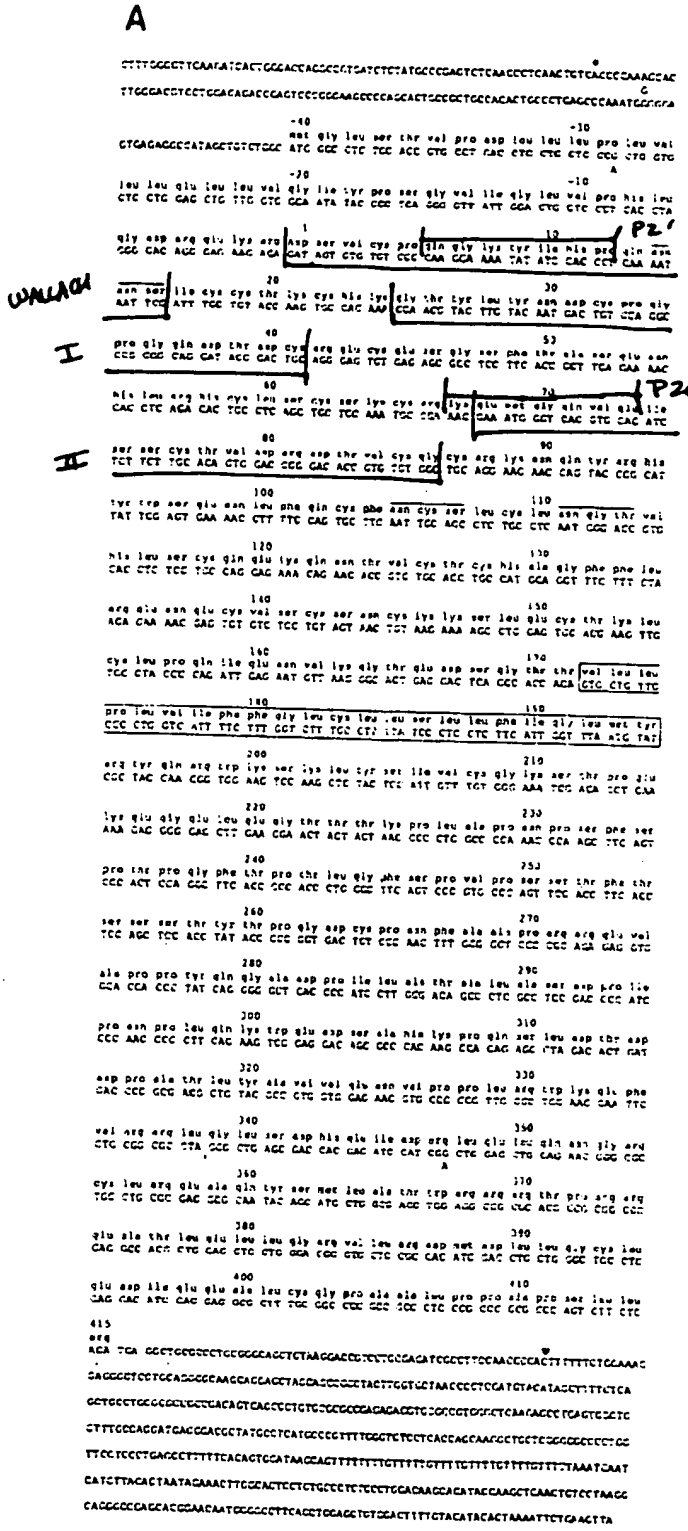
TNF-R Is Related to Nerve Growth Factor Receptor and the B Cell Activation Molecule Bp50

Cysteine-rich repeats have been detected in the extracellular domains of the EGF precursor and EGF receptor (Doolittle et al., 1984; Ullrich et al., 1984) and LDL receptor (Yamamoto et al , 1984), but the TNF-R sequence reveals no significant amino acid homologies with these molecules. However, cysteine-rich extracellular domains have also been reported in the nerve growth factor receptor (NGF-R) (Johnson et al., 1986) and the B lymphocyte activation molecule Bp50 (CDw40) (Stamenkovic et al., 1989). These molecules have significant homology to the TNF-R in their extracellular domains (Figure 3). All three molecules share a similar set of cysteine-rich subdomains. Optimal alignment of the sequences for the extracellular domains of TNF-R, NGF-R, and Bp50 shows the close conservation of the cysteine residues and an overall identity of 29% between TNF-R and NGF-R and 24% between TNF-R and Bp50 in 167 residues (Figure 3). No significant homology is seen in the transmembrane or intracellular domains of these molecules.

TNF-R Transcript Is Expressed in a Variety of Cell Types

A panel of human cells and tissues was examined for the presence of TNF-R mRNA. Figure 4A shows expression of TNF-R mRNA in human term placenta and adult liver, as well as the breast carcinoma MB436, the nontumorigenic transformed breast epithelial cell line HBL100, the glioblastoma A172, and a primary squamous carcinoma, FG. In addition, cell lines resistant and sensitive to TNF cytotoxicity were tested for TNF-R mRNA. TNF-resistant cell lines (T-24 bladder carcinoma, A549 lung carcinoma) and TNF-sensitive cell lines (MCF-7 breast carcinoma and ME-180 cervical carcinoma) all exhibit TNF-R mRNA (Figure 4B).

The increased expression of TNF-Rs on cells after treatment with interferon-γ has been reported (Aggarwal et al., 1985). To test whether the levels of transcript for the TNF-R



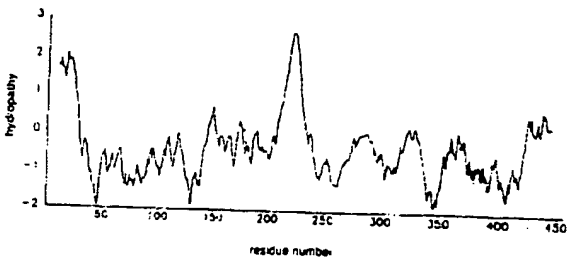


Figure 1. Human TNF-R cDNA and Amino Acid Sequences and Hydropathy Plot

(A) Composite nucleotide and deduced amino acid sequence of TNF-R cDNA. Amino acids are numbered in the sequence. The composite of HL-60 clones begins at the first nucleotide and ends at nucleotide 1607 (▼). The placental clone begins at nucleotide 67 (●) and ends at the last nucleotide. Three nucleotide differences at positions 75, 217, and 1342 are noted, with the top nucleotide representing that found in the placental clone and the bottom representing the HL-60 sequence. Aspartic acid (Asp) is the initial residue in the N-terminal sequence obtained from native TNF BP in human serum and is labeled as position 1. Amino acids −40 to −1 comprise presumed signal peptide and proteolytically processed N-terminal residues. Overlines represent three potential N-linked glycosylation sites (residues 14, 105, and 111). The predicted transmembrane region (residues 172–194) is boxed.
(B) Hydropathy profile of protein sequence in (A).

-β, we treated A549 cells with these cytokines for 24 hr prior to harvest of the mRNA. Levels of TNF-R mRNA are relatively constant after treatment with these cytokines alone or in combination (Figure 4C). Thus, if interferon-γ or TNF regulation of receptor does occur in A549 cells, it does not appear to be transcriptional or it is mediated through another class of receptor. We do not know whether TNF-R mRNA is affected by these cytokines in other cell types.

TNF-R message was also found in several hematopoietic cell lines. The cultured T cell lines CEM, HSB, and HuT 78, the functional cytotoxic T cell line PM, and the

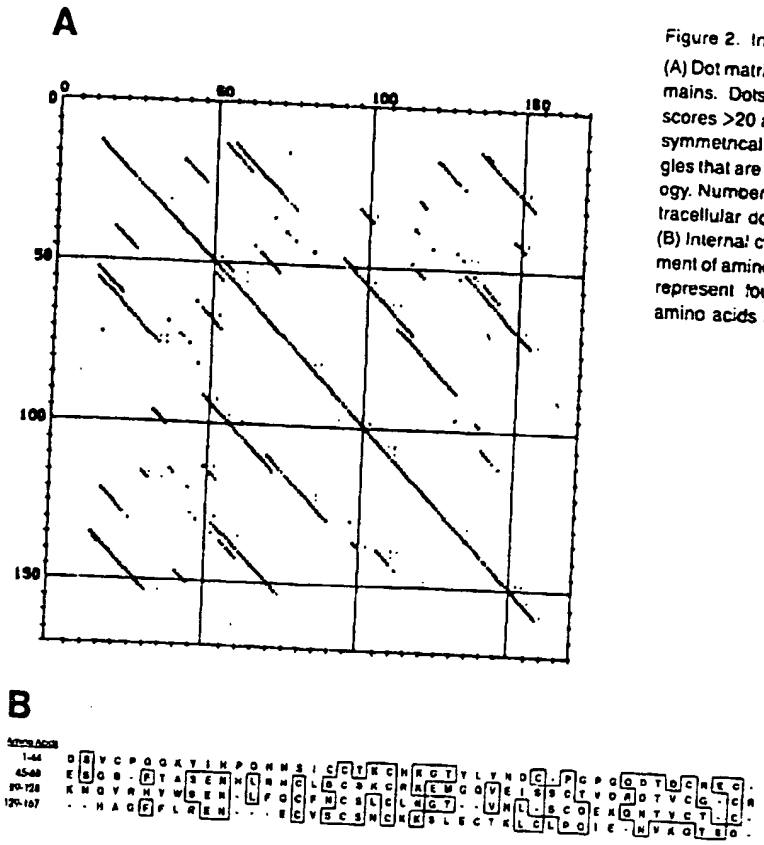


Figure 2. Internal Repeats in the TNF-R

(A) Dot matrix plot of internal homologies in the TNF-R extracellular domains. Dots are placed where Dayhoff mutation matrix alignment scores >20 are obtained (Dayhoff et al., 1983). The plot is necessarily symmetrical around the diagonal line of identity; other lines at 45° angles that are off of the main diagonal represent areas of internal homology. Numbers denote amino acid positions in the predicted mature extracellular domain.

(B) Internal cysteine repeats in the TNF-R extracellular domain. Alignment of amino acid sequences in the extracellular portion of the TNF-R represent four internal subdomains. Identical residues are boxed; amino acids are numbered in the left margin.

erythroleukemia line K562 have a single detectable species of TNF-R mRNA (data not shown). The cultured B cell line RPMI-1788, but not the EBV-transformed B cell lines JY, LB, and BOC, have TNF-R mRNA (data not shown). Uninduced U-937 cells were found to have relatively high levels of TNF-R mRNA, and uninduced HL-60 cells contained markedly less (data not shown). Thus, message for the cloned TNF-R seems widely though not ubiquitously expressed. In all cells that express TNF-R, a single species of mRNA of about 3.0 kb is detected. This suggests that the cDNA clones we obtained do not represent complete copies of the TNF-R mRNA; some nucleotide sequences in the untranslated regions are missing.

Transfection and Expression of the Human TNF-R cDNA

The 2.1 kb placental cDNA clone was inserted into the mammalian expression vector pRK5. This cDNA starts at nucleotide position 64 (Figure 1A), with the initiating me-

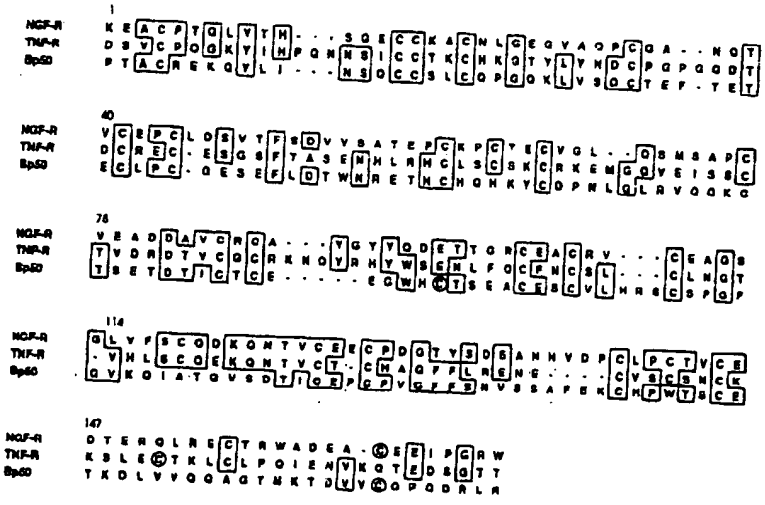


Figure 3. Homology in the Extracellular Domains of NGF-R, TNF-R, and Bp50

Optimized alignment of the protein sequence of the extracellular domains of NGF-R, TNF-R, and Bp50 (CDw40) is shown with gaps introduced to optimize matches. Identical amino acids are boxed. Cysteine residues not conserved among all three sequences are circled. Residues are numbered in relation to their position in TNF-R.

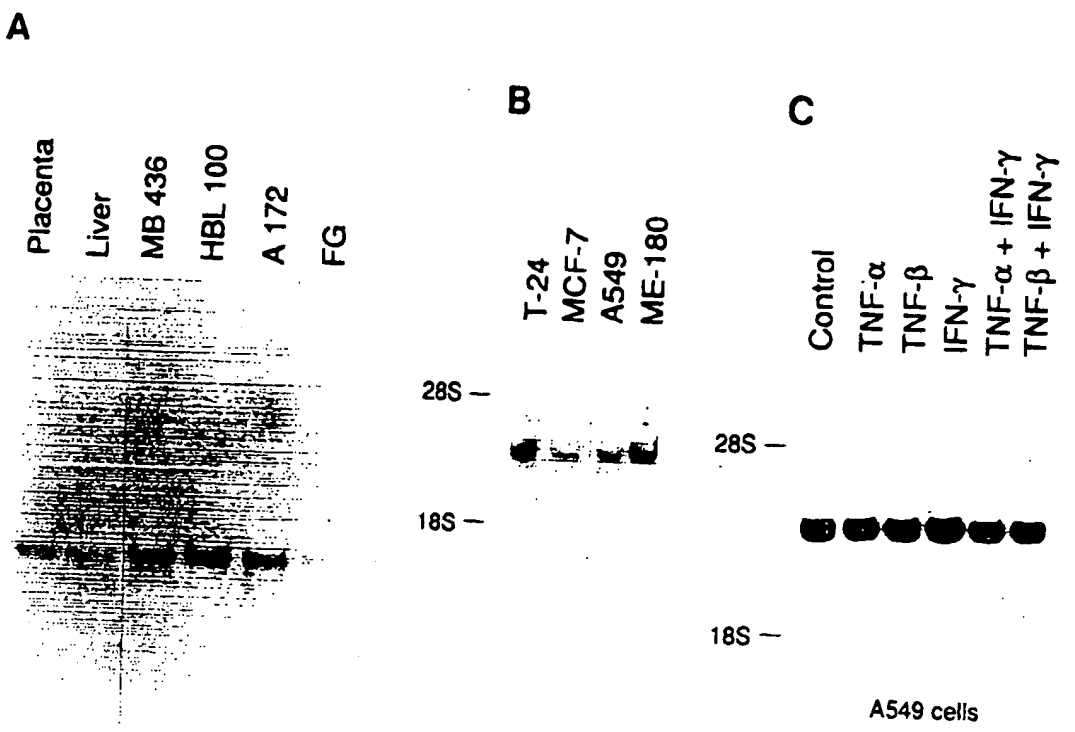


Figure 4. Northern Blot Analysis of TNF-R mRNA

(A) Northern analysis of TNF-R mRNA in placenta, liver, and transformed cell lines. Northern blot analysis of 4 μg of poly(A)$^+$ RNA from normal human term placental and adult liver tissues, the human transformed cell lines MB436, HBL100, and A172, and the primary squamous carcinoma FG.
(B) Northern blot analysis of both TNF-resistant and TNF-sensitive cell lines. TNF-resistant cell lines: A549 and T-24. TNF-sensitive cell lines: MCF-7 and ME-180. There is 3 μg of poly(A)$^+$ RNA per lane. The positions of ribosomal RNA bands are denoted as 28S and 18S.
(C) Levels of TNF-R mRNA in A549 cells after IFN-γ or TNF treatment. There is 7 μg of poly(A)$^+$ RNA per lane from A549 cells after treatment with either TNF-α, TNF-β, or interferon γ, or both (0.1 μg/ml each) for 24 hr prior to harvest of mRNA. Control lane: untreated cells.

thionine 118 bp downstream (position 182, Figure 1A). The cDNA is under the transcriptional control of the cytomegalovirus immediate-early promoter and is followed by the SV40 termination and polyadenylation signals. The TSA 201 cell line, a subclone of the human embryonic kidney cell line 293s (Graham et al., 1977), which constitutively expresses large T antigen, was selected for transient transfection experiments because of its high transfection efficiency and low numbers of endogenous TNF-Rs. Following transfection with the TNF-R expression construct, cells were tested for the ability to bind specifically either biotinylated or ^{125}I-labeled TNF-α.

An increase in the relative capacity of pRK-TNF-R-transfected cells to bind biotinylated TNF-α can be seen using fluorescence-activated cell sorting (FACS). Mock-transfected TSA 201 cells display a low level of binding of biotinylated TNF-α, presumably to endogenous receptors (Figures 5A and 5B). The levels of TNF-α binding are substantially increased on cells transfected with the TNF-R construct, as shown by the shift to the right of the fluorescence histogram (Figure 5F). The intensity of fluorescent staining is reduced to background levels in both populations by preincubation of cells with either nonbiotinylated TNF-α or TNF-β (Figures 5C, 5D, 5G, and 5H), demonstrating that the observed binding is specific.

A saturation isotherm for the specific binding of ^{125}I-TNF-α was performed by sequential dilution of the specific activity of the radioligand with unlabeled TNF-α at concentrations ranging from 67 pM to 33 nM (Figure 6A). The specific binding of ^{125}I-TNF-α is saturable, and the Scatchard analysis of these data using nonlinear least-squared regression reveals two binding sites ($p < 0.05$; Figure 6A, inset) with high (K_d = 0.56 nM) and low (K_d = 19.6 nM) affinity. The number of binding sites for these two receptor subtypes on the transiently expressing TSA 201 cells is ~50,000 and 630,000 sites per cell, respectively. ^{125}I-TNF-α binding to mock-transfected control cells was at least 10-fold lower than pRK-TNF-R-transfected cells, suggesting low numbers of endogenous receptors. Increasing concentrations of unlabeled TNF-β cause a dose-dependent decrease in specific ^{125}I-TNF-α binding in a manner very similar to that that seen with unlabeled TNF-α (Figure 6B). This suggests that the TNF-R expressed on the transfected cells recognizes TNF-α and TNF-β with approximately equal affinity.

Discussion

The data presented here describe the cloning and expression of a receptor for human TNF-α and -β. The deduced amino acid sequence of the TNF-R reveals a structure typical of cell surface receptors for polypeptides: it contains a signal peptide and extracellular, transmembrane, and intracellular regions. There is a significant degree of homol-

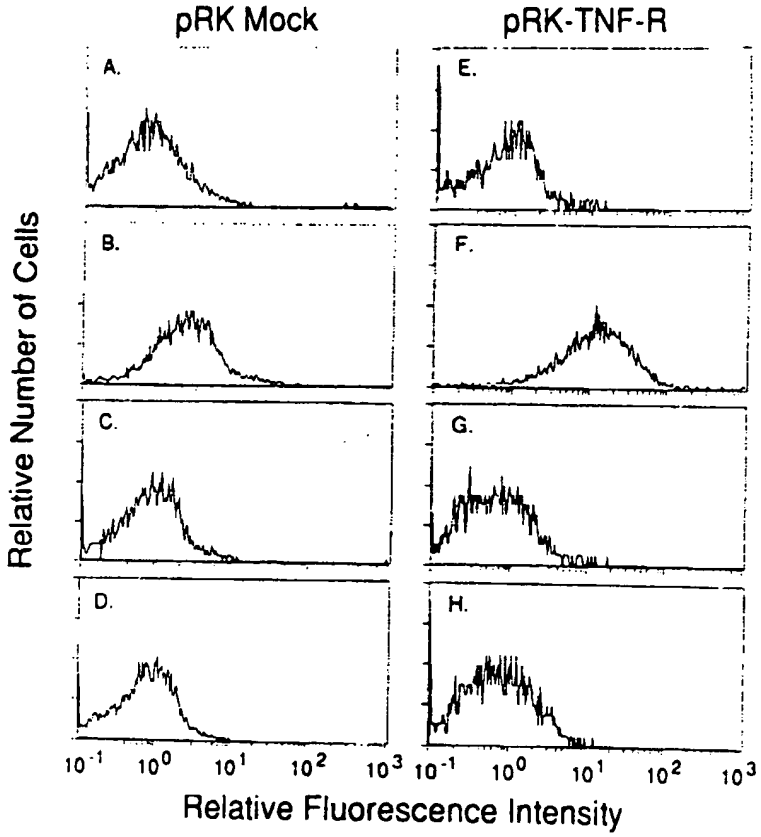


Figure 5. FACS Analysis of pRK-TNF-R-Transfected TSA 201 Cells

The left column contains histograms from mock pRK5-transfected cells; the right column contains histograms from pRK-TNF-R-transfected cells. Cells were stained with PE-conjugated streptavidin after pretreatment with or without biotinylated TNF-α (b-TNF-α). (A) and (E), PE-streptavidin without b-TNF-α; (B) and (F), PE-streptavidin plus b-TNF-α; (C) and (G), preincubation of the cells with a 5-fold excess of unlabeled TNF-α prior to staining with PE-streptavidin plus b-TNF-α; (D) and (G), preincubation with a 2-fold excess of unlabeled TNF-β prior to staining with PE-streptavidin plus b-TNF-α.

ogy between the TNF-R and the NGF-R, as well as the B lymphocyte activation molecule Bp50, suggesting that they comprise a family of related growth factor receptors. The four 40 amino acid repeats of the extracellular portions of these receptors may form relatively independent folded subdomains, each tightly cross-linked by disulfide bonds. Because these cysteine-rich domains form virtually all of the extracellular portion of the TNF-R, it is likely that they contain the ligand binding site(s), although it is not immediately clear how the biologically active TNF trimer binds the receptor. In this regard it is interesting to note that the net change of the extracellular domain of the TNF-R is positive, while that of its ligand is negative. The converse is true for NGF-R and its ligand, where the extracellular domain is strongly acidic and the ligand is basic. This would suggest that electrostatic attractions play an important role in the interactions of these related receptor molecules with their respective ligands. One possibility is that the cysteine repeat units form a general structural framework that, depending upon specific amino acid substitutions, could be varied to accommodate a variety of ligands.

The mechanisms of signal transduction by TNF and its receptor are obscure. The intracellular domain, though large enough to possess an enzymatic activity or to interact with other proteins that may mediate signal transduction, has no apparent sequence homology with any proteins in the available data bases. The cytoplasmic domain is rich in serine, threonine, and tyrosine but shows no homology to the catalytic domain of the tyrosine or serine/threonine-specific protein kinases. However, there is sequence similarity to the canonical phosphorylation sites (Ser/Thr-X-Arg/Lys) that can be acted upon by protein kinase C (Woodget et al., 1986) at amino acid positions 223, 366, and 371. Also present are a potential cyclic nucleotide-dependent protein kinase phosphorylation site at amino acid 368 (Feramisco et al., 1980) and a consensus tyrosine kinase phosphorylation site at residue 354 (Patchinsky et al., 1982). The binding of TNF to its receptor has been shown to increase both GTP binding and GTPase activity in HL-60 membrane preparations, leading to the suggestion that a GTP binding protein might couple TNF-induced signaling to biological effects (Imamura et al., 1988). However, the TNF-R has no homology to other receptors that are known to interact with G proteins.

Internalization of the native TNF-R in the absence of ligand has been shown (Smith et al., 1990; Ding et al., 1989) and shown to occur more rapidly in the presence of ligand (Smith et al., 1990; Imamura et al., 1987). While a majority of reports indicates the degradation of receptor after internalization (Watanabe et al., 1988; Smith et al., 1990), one suggests that the receptor is continuously recycled (Vuk-Pavlovic and Kovach, 1989). These differences could represent cell type–specific differences or differences in processing for distinct types of TNF-R. Regions of the intracellular domain of the cloned TNF-R reported here are extremely rich in proline, glutamic acid, serine, and threonine. The presence of these so-called PEST sequences has been shown to correlate with rapid intracell-

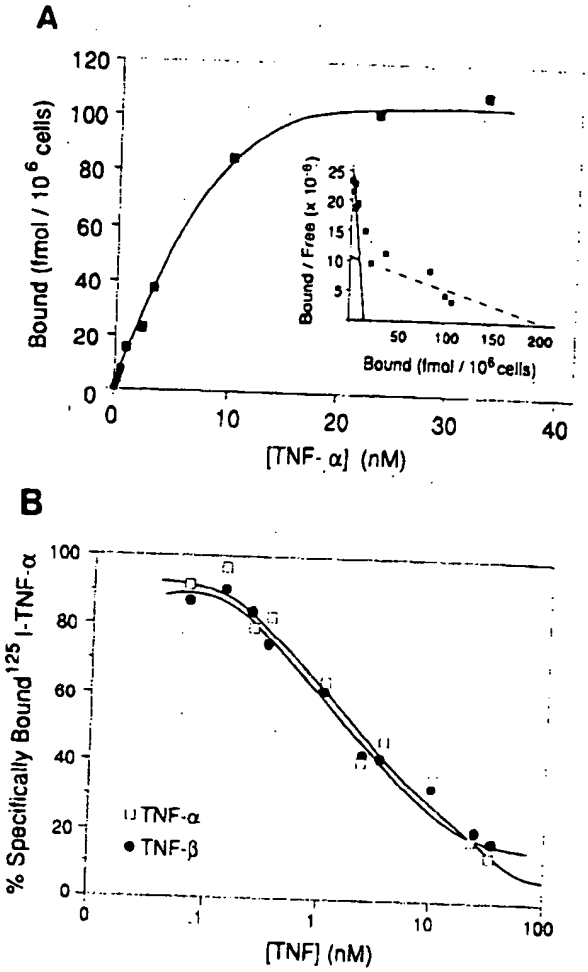


Figure 6. Binding Characteristics of Recombinant Human TNF-R Expressed in TSA 201 Cells

(A) Saturation isotherm of specific binding of ^{125}I-labeled TNF-α on transfected cells. Replicate samples of TSA 201 cells transiently expressing pRK-TNF-R were incubated with ^{125}I-TNF-α (16.7 pM) alone or in the presence of increasing amounts of unlabeled TNF-α, and the specific binding of ^{125}I-TNF-α was determined at each concentration. The inset presents the data transformed by Scatchard analysis. These results were from a single experiment that had been repeated three times with either triplicate or duplicate determinations.

(B) Displacement curves showing inhibition of the specific binding of ^{125}I-TNF-α by unlabeled TNF-α (□) or TNF-β (●). The experiment was performed as described in (A), with ^{125}I-TNF-α displaced with increasing amounts of TNF-α or -β.

lar protein degradation (Rogers et al., 1986). This leads to the speculation that the receptor encoded by this gene has a high turnover rate in its native state. This speculation is consistent with our observations that A549 cells have relatively high levels of mRNA for the TNF-R yet relatively low numbers of cell surface receptors (unpublished data).

It is not clear that the effector functions of TNF are achieved by transduction of a signal via the receptor after ligand binding. A direct intracellular role for TNF in cytotoxicity has been proposed based on TNF microinjection experiments (Smith et al., 1990), although using other cell lines we have not been able to demonstrate activity for microinjected TNF (D. Pennica and D. V. G., unpublished data). If internalization of the ligand is the important step in the biochemical action of TNF, the receptor may play no more a role than transporting TNF to the inside of the cell. However, the fact that antibodies generated against soluble TNF BPs will cross-react with cell surface molecules and act as TNF agonists suggests that TNF-R signal induction can occur without internalization of the TNF (Engelmann et al., 1990).

Shedding the extracellular domain of the TNF-R might be used as a protective mechanism by cells to avoid the cytotoxic effects of TNF. The presence of soluble TNF BP in the serum of cancer patients may represent a mechanism by which tumors evade host anti-tumor defenses by modulating systemic levels of TNF. Soluble forms of the receptor for IL-2 are released from activated human lymphoid cells (Rubin et al., 1985), and its levels are found increased in bodily fluids in disease states (Marcon et al., 1988). Soluble receptors for IL-6 and IFN-γ have recently been detected in human urine (Novick et al., 1989), soluble truncated forms of the NGF receptor have been seen in human urine and amniotic fluid (Zupan et al., 1989), and a cDNA that encodes a soluble form of murine IL-4 has been reported (Mosley et al., 1989). The prevalence of such soluble receptors suggests a normal regulatory role for these molecules. Their mode of action could be to limit the amount of available cytokine by binding it in solution, thus preventing the cytokine from reaching its cell surface target. TBP I, the soluble TNF-BP that shares N-terminal homology to the TNF-R reported here, binds TNF-α with greater affinity than TNF-β (Engelmann et al., 1990). Our studies show that TNF-R binds TNF-α and -β with approximately equal affinities. This suggests that solubilization of the extracellular domain of the receptor may induce a change in the relative affinities of the binding component for TNF-α and TNF-β.

Most if not all mammalian cells appear to have receptors for TNF. However, the number of TNF-Rs per cell is relatively low. In the 5′ untranslated region of TNF-R mRNA two short open reading frames are seen, one of 43 codons and one of 3 codons. Such short open reading frames are not uncommon in growth factor receptor RNAs. They are found 5′ of the main open reading frame in the GM-CSF R (Gearing et al., 1989), the human IL-6 R (Yamasaki et al., 1988), the murine IL-1 R (Sims et al., 1988), and the human IL-2 R (Nikaido et al., 1984; Hatakeyama et al., 1989). It has been postulated that these short open reading frames might act, if translated, to dampen the translation of the main receptor coding regions (Gearing et al., 1989). Such a mechanism might partly explain the low numbers of these receptors, in relation to the levels of mRNA, on normal cell types.

Interpretation of published data from affinity labeling cross-linking studies is made difficult because cross-linked ligand is itself resolved into monomers, dimers, and trimers by SDS–PAGE, but estimates of the size of cell surface TNF-Rs is generally between 55 and 138 kd (Creasey et al., 1987; Stauber et al., 1988; Hohmann et al., 1989). Receptors of considerably larger size, up to 310 kd, have also been reported (Smith and Baglioni, 1989), but these forms might reflect receptors cross-linked to associated regulatory proteins, or even complexes of cross-

linked receptors. Association with other proteins or "adaptor subunits" has been shown for other receptors, most notably IL-2 R (Teshigawara et al., 1987; Hatekeyama et al., 1989) and IL-6 R (Yamasaki et al., 1988). In addition, NGF-R is thought to require association with another molecule for high-affinity binding of its ligand (Radeke et al., 1987). Association with an as yet unknown protein may be required for the TNF-R reported here to bind ligand with high affinity. One possible explanation for the two binding sites observed in the cells transfected with pRK-TNF-R is that a small number of the transiently expressed receptor proteins interacts with an endogenous protein present in a limited quantity within the TSA 201 cell. This subpopulation of receptors might then bind ^{125}I-TNF-α with a higher relative affinity (K_d = 0.66 nM), while the bulk of the expressed receptors (92% based on B_{max} predictions) binds ligand with ~30-fold lower affinity.

The relationship between the TNF-R described here and other potential TNF-Rs is not clear. The predicted 415 amino acid molecule we identified by cDNA cloning encodes a protein with a predicted M_r of 50,578. Since there are three potential N-linked glycosylation sites, and since protein biochemical studies consistently reveal carbohydrate content on the TNF-R molecules analyzed, it is likely that the apparent M_r of the TNF-R reported here is greater than 50,000 in its native state. The recent report of two immunologically distinct forms of TNF BPs in urine, one of which has N-terminal sequence homology to the sequence reported here (Engelmann et al., 1990), argues for the existence of at least two cell surface TNF-Rs that shed their extracellular domains. This is consistent with the report of two major types of TNF-R on different cell types (Hohmann et al., 1989), suggesting that the molecule reported probably represents one of the two types of cell surface TNF-R. The mechanisms by which the extracellular domains of the TNF-R are shed are not known. Soluble IL-4 receptor is thought to be the result of alternative splicing of IL-4 mRNA (Mosley et al., 1989). In contrast, the presence of only one detected species of mRNA for this TNF-R suggests that the soluble form is generated proteolytically by cleavage of the extracellular domain from the cell surface receptor. Soluble TNF BP may be resistant to subsequent proteolysis after release as a result of a compact disulfide-bonded structure. The physiological significance of this process remains unknown. The availability of this and other cloned TNF-R cDNAs should allow for the resolution of these issues as well as the elucidation of many of the complexities of the multiple activities of TNF.

Experimental Procedures

Purification of Serum TNF BP and Amino Acid Sequencing of Proteolytic Fragments

TNF-α affinity chromatography fractions of serum proteins from human cancer patients were shown to inhibit the activity of TNF-α and TNF-β (Gatanaga et al., 1990). These fractions were electrophoresed on SDS–PAGE and found to contain several components. Samples from the affinity column were loaded directly onto a small glass column (1.5 mm × 50 mm) packed with 15 μm of C-18 packing material (J. T. Baker) and eluted on a HP 1090 HPLC with a linear gradient of 1%–60% acetonitrile in 0.1% TFA and water at a flow rate of 0.2 ml/min. Eluted peaks were sequenced directly on a prototype sequencer (U.S. patent number EP0257735), and sequences obtained were compared to the protein sequences in the available data bases using the DFASTP program. An early eluting peak, M_r = 28,000, produced the only unknown sequence. Further internal sequence of this protein was obtained by digesting the purified material with a 1:10 ratio (enzyme to substrate) of lysyl endopeptidase (Wako Chemicals) at pH 8.0 in the presence of 0.05% SDS, 0.1 M Tris–HCl at 37°C for 18 hr. The digested peptides were then separated on HPLC as above. The two major eluting peaks, PF I and PF II, were sequenced as above.

cDNA Cloning

Two λgt10 libraries, a placental cDNA library prepared as described (Ullrich et al., 1985), and a random primed cDNA library made from the promyelomonocytic cell line HL-60 (provided by Karen Fisher) were probed with two oligonucleotide probes derived from the protein sequences of PF I and PF II using human codon bias (Lathe, 1985). The probes, 5′-AAGGGCACCTACCTGTACAATGACTGCCCTGGCTTTGGCCAGGATGAGAATGA from PF I and 5′-AAGGAGATGGGCCAGGTGGAGATCTCCTCCTGCACAGTGGAC AATGACACAGTGTGTGG from PF II, were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase and used to screen replicate sets of nitrocellulose filters containing ~1 × 10^6 independent clones from each of the two libraries. Filters were probed at 42°C in a hybridization solution that was 20% formamide, 0.1% SDS, 5× Denhardt's solution, 50 μg/ml salmon sperm DNA, 50 mM $NaPO_4$, 0.1% sodium pyrophosphate. Filters were washed twice in 0.5× SSC, 0.1% SDS at 42°C. Hybridizing phage were plaque purified, DNA was prepared, and cDNA inserts were isolated and subcloned using standard techniques (Maniatis et al., 1982). Four clones from the HL-60 library, HL-60-2, -3, -10, and -14, and one clone from the placental library were sequenced on both strands using the chain termination procedure (Sanger et al., 1977).

Northern Analysis of TNF-R mRNA

Northern hybridization was performed as previously described (Thomas, 1980; Wong et al., 1988). Briefly, total cytoplasmic RNA was extracted from cells, enriched for poly(A)$^+$ mRNA, electrophoresed on a formaldehyde-agarose (1.2%) gel, and transferred to nitrocellulose. The filters were baked for 30 min at 80°C under vacuum and hybridized to a ^{32}P-labeled TNF-R probe for 16 hr. The probe consisted of the cDNA insert isolated from the placental clone (2.1 kb EcoRI fragment) labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP by the random priming method. Filters were washed at 60°C in 0.1× SSC, 0.1% SDS for 30 min. Autoradiography was carried out for 24 hr using Kodak XAR-5 film and an intensifying screen.

TNF-R Expression Plasmid and Transfection of TSA 201 Cells

A 2.1 kb TNF-R cDNA fragment was isolated by a partial EcoRI digest from the placental phage TNF-R clone. This fragment was ligated into the EcoRI site of the expression plasmid pRK5 (R. Klein and D. V. G., unpublished data). The cDNA in the expression construct, pRK-TNF-R, is downstream of the cytomegalovirus promoter/enhancer and under its transcriptional control. Downstream of the cDNA insert are SV40 termination and polyadenylation signals. Human TSA 201 cells (obtained from R. DuBridge) are a derivative of the human embryonic kidney cell line 293s (Graham et al., 1977), which constitutively expresses large T antigen. These were transfected with either the pRK-TNF-R expression plasmid or mock transfected with the pRK plasmid without a cDNA insert. Transfections were performed in 100 mm plates using 7.5 μg of plasmid DNA per plate by the calcium phosphate precipitation method essentially as described (Gorman, 1985), except that the precipitates were left on the cells for 16–18 hr, and the cells were not shocked with DMSO in PBS. The transfected cells, transiently expressing TNF-R, were assayed 48 hr after transfection.

Analysis of Transfected TSA 201 Cells

For FACS analysis, TNF-α was biotinylated using biotin-N-hydroxysuccinimide ester at a 1:2.5 ratio of biotin ester:protein as described (Ranges et al., 1988). Forty-eight hours after transfection, cells (10^5) were treated with 50 nM biotinylated TNF-α by incubation for 2 hr at 4°C in PBS + 2% fetal calf serum (FCS). After washing twice the cells were stained for 30 min at 4°C with phycoerythrin (PE)-conjugated streptavidin and then washed twice and resuspended in PBS + 2%

FCS containing 0.5 μg/ml propidium iodide. For competition binding with unlabeled TNF-α and TNF-β, cells were preincubated with either 250 nM nonbiotinylated TNF-α or 100 nM nonbiotinylated TNF-β prior to staining with PE-conjugated streptavidin plus 50 nM biotinylated TNF-α. The cells were analyzed on a Coulter Elite Flow Cytometer using the 488 nm line of an argon ion laser, gating only on propidium iodide–excluding cells. PE emission was detected using a 575 nm (± 25 nm) band pass filter, and propidium iodide was detected with a 680 nm long pass filter. Electronic compensation was used to subtract the spectra overlap of PE into the propidium iodide detector.

For analysis of binding of ^{125}I-TNF to transfected cells, pRK-TNF-R or pRK mock-transfected cells were harvested with PBS containing 1 mM EDTA and washed with PBS containing 0.1% BSA and 0.02% sodium azide (PBSA buffer). Duplicate or triplicate samples of 2–2.5 × 10^6 cells in 0.5 ml of PBSA were incubated at 4°C for 2 hr while shaking with 16.7 pM ^{125}I-TNF-α (New England Nuclear: 88.6 mCi/mg) alone or in the presence of increasing concentrations of unlabeled recombinant human TNF-α or TNF-β (rHuTNF-α and -β, Genentech). Nonspecific binding was determined by the addition of 0.33 mM unlabeled TNF-α. Cells were centrifuged at 14,000 × g for 15 min, and unbound ^{125}I-TNF-α was aspirated. The cell pellet was washed once with 1 ml of ice-cold PBSA. The amount of ^{125}I bound was determined by counting the cell pellets in a gamma counter. The data were fit using nonlinear least-squared regression analyses according to Marquardt algorithms (GraphPAD Inplot version 3.0, GraphPAD Software, San Diego, CA).

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Appendix J

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A Tumor Necrosis Factor-binding Protein Purified to Homogeneity from Human Urine Protects Cells from Tumor Necrosis Factor Toxicity*

(Received for publication, December 7, 1988)

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Unfractionated preparations of the proteins of human urine provided protection against the *in vitro* cytocidal effect of tumor necrosis factor (TNF).

In certain cells, the proteins decreased expression of the receptors for TNF in a temperature-dependent way.

In all cells examined, the proteins were found to interfere also with the binding of both TNF and interleukin-1 when applied directly into the binding assays. That effect could be observed in the cold, suggesting that it was independent of cellular metabolism.

A protein which protects cells against the cytotoxicity of TNF was purified from human urine by chromatography on CM-Sepharose followed by high performance liquid chromatography on Mono Q and Mono S columns and reversed phase high performance liquid chromatography. This protein is a very minor constituent of normal urine, with an apparent molecular weight of about 27,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis under both reducing and nonreducing conditions. Homogeneity of the purified protein was confirmed by microsequence analysis which revealed a single N-terminal sequence: Asp-Ser-Val-Cys-Pro-. The protein protected cells from TNF toxicity at concentrations of a few nanograms per ml and interfered with the binding of both TNF-α and TNF-β to cells, when applied simultaneously with the cytokines. However, unlike crude preparations of the urinary proteins, the purified protein did not induce in cells a decrease in ability to bind TNF nor did it interfere with the binding of interleukin-1 to its receptor. Direct, specific binding to the protein of TNF-α and, to a lesser extent, also TNF-β, but not of interleukin-1 nor interferon-γ could be demonstrated. It is suggested that this protein blocks the function of TNF by competing for TNF with the TNF receptor and not by interacting with the target cell.

Tumor necrosis factor (TNF)[1] is outstanding among the various mediators of immune defense in the extent to which it may cause harm to the host. Although it is effectively protective against various pathogens, this cytokine also has a mediating role in the pathological manifestations of diseases, including those caused by these very pathogens against which TNF can protect (for review, see Refs. 1 and 2). Exploring ways for suppressing the formation of TNF and antagonizing its destructive potential seems, therefore, of just as much practical importance as defining ways to take advantage of the beneficial effects of TNF in therapy.

Human urine has been shown to contain proteins which can interfere with the function of interleukin-1 (IL-1) (3-5). In view of the marked similarity in the physiological function of TNF and IL-1, we have posed the question whether proteins found in urine can also suppress the activity of TNF. We report here the purification to homogeneity and initial characterization of a protein which is present in human urine in minute amounts. This protein binds TNF, thus preventing its interaction with the TNF receptors and blocking its activity. It does not interfere with the binding of IL-1 to cells and differs in this, as well as in some other characteristics, from uromodulin, a urinary protein which suppresses the function of IL-1 (5-7) and, according to a recent study (7), also binds TNF with a high affinity, although it is apparently unable to interfere with its function. During preparation of this manuscript a study by Seckinger *et al.* (8) was published, describing a urinary antagonist to TNF which may be identical to the one described here.

MATERIALS AND METHODS

Cells

Murine A9 cells (9) and human foreskin fibroblasts, FS11 (established in our laboratory by Dr. D. Rotman), were cultured in Dulbecco's modified Eagle's minimal essential medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml amphotericin B. The media were supplemented with 10% newborn calf serum for the A9 cells and with 10% fetal calf serum for the FS11 cells.

Cytokines

Recombinant human TNF-α (rhuTNF-α, 6×10^7 units/mg protein), recombinant murine TNF-α (rmuTNF-α, 2.6×10^7 units/mg protein), and recombinant human TNF-β (rhuTNF-β, lymphotoxin, 1.2×10^8 units/mg protein) were kindly provided by Dr. G. Adolf, Boehringer Institute, Vienna, Austria. Recombinant human IL-1α (rIL-1α, 2.5×10^8 units/mg protein), consisting of the 154 carboxyl-

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‡ Recipient of a fellowship from the Minerva Foundation (Federal Republic of Germany).

§ Holder of the Maurice and Edna Weiss Chair in Interferon Research.

¶ To whom correspondence and reprint requests should be ad-

[1] The abbreviations used are: TNF, tumor necrosis factor; CUP, crude urinary proteins; IFN, interferon; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TBP, TNF-binding protein; SDS, sodium dodecyl sulfate; IL-1, interleukin-1; PBS, phosphate-buffered saline; r, recombinant; hu, hu-

Appendix K

terminal amino acids of the 271-amino acid human IL-1 precursor, was a gift of Drs. A. Stern and P. T. Lomedico (Hoffmann La Roche, Nutley, NJ). Recombinant human interferon-γ (rIFN-γ) (5×10^7 units/mg protein) was provided by Dr. D. Novick of our laboratory.

Radiolabeling of Cytokines

All cytokines were labeled with ^{125}I by the chloramine-T method, as previously described (10). The amounts of incorporated label in the preparations of cytokines used in this study were as follows: 112 μCi/μg protein for rmuTNF-α, 126 μCi/μg protein for rhuTNF-α, 113 μCi/μg protein for rhuTNF-β, and 139 μCi/μg protein for rIL-1-α. The purified TNF-binding protein (TBP) was labeled by the same method to a specific activity of 238 μCi/μg protein.

Assays for the TNF-binding Protein

Quantitation of the Protective Effect of the TBP against TNF Cytotoxicity—Mouse A9 cells were seeded in 96-well microtiter plates at a density of 15,000–20,000 cells/well. Urinary protein samples were applied, about 24 h later, together with cycloheximide (50 μg/ml) and rhuTNF-α (5 units/ml), and the cells were further incubated at 37 °C for 14 h. Cell viability was then quantitated by the neutral red uptake assay (11). For maximal sensitivity, the test was initiated when the A9 cells were just about to reach confluency. One unit of protective activity was defined as the amount of TNF-binding protein in whose presence the number of cells remaining viable, under the conditions of the assay, was doubled. The morphology of the A9 cells when protected from TNF toxicity by TBP is shown in Fig. 1.

Quantitation of the Binding of Cytokines to Cells and Its Decrease by Proteins of the Urine—A9 and FS11 cells were seeded into 15-mm tissue culture plates at a density of 2.5×10^6 cells/well. After 24 h incubation at 37 °C in 5% CO_2 atmosphere, the plates were transferred to ice, the growth medium was removed, and the radiolabeled cytokines (10^6 cpm/plate, counting efficiency 50%) were introduced in 150 μl of ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$ containing 0.5% bovine serum albumin and 0.1% NaN_3 (PBS/BSA)). Following incubation for 2 h on ice, the cells were rinsed twice with ice-cold PBS/BSA and detached with Ca^{++}- and Mg^{++}-free PBS containing 5 mM EDTA. The cell-associated radioactivity was determined using a γ-counter. Nonspecific binding of the cytokines, determined by adding 1000-fold excess of unlabeled cytokine, was subtracted from all values. Effects of urinary proteins on the binding of cytokines were examined by either applying the proteins directly into the binding assay or pretreating the cells by the proteins, for various durations, either at 37 °C (in growth medium) or at 4 °C (in PBS/BSA) and then removing the proteins prior to the quantitation of cytokine binding. Samples of the proteins were tested in duplicates.

Solid Phase Assay for the Binding of Various Cytokines to the Purified TNF-binding Protein—PVC 96-well radioimmunoassay plates (Dynatech 1-220-25) were coated with rhuTNF-α, rhuTNF-β, rIL-1α, or rIFN-γ by incubation, for 12 h at 37 °C, with solutions of 5 μg/ml of the pure cytokines in PBS containing 0.02% NaN_3. The wells were then rinsed and incubated further, for 8 h at 4 °C, with PBS containing 0.5% BSA, 0.02% NaN_3, and 0.05% Tween-20 (blocking solution). Samples of radiolabeled TBP (10^5 cpm in 50 μl of blocking solution) were then applied, either alone or in the presence of various cytokines or excess unlabeled TBP, and the plates were incubated for 12 h at 4 °C. They were then rinsed three times with blocking solution. The counts of the material which remained bound to the PVC plates were determined using a γ-counter.

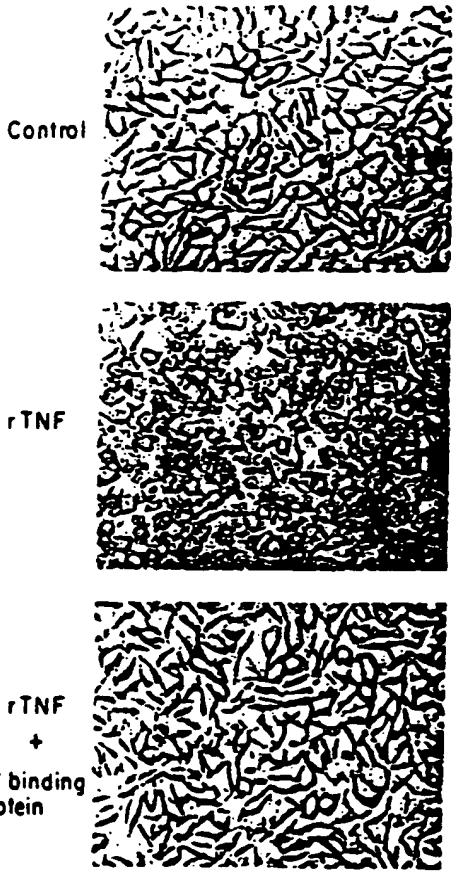

FIG. 1. The cytocidal effect of TNF in A9 cells and its decrease by the urinary proteins. Photographs of cells used for the bioassay of the protective effect of the urinary proteins (see "Materials and Methods") were taken after 14 h treatment. *Top panel*, control, cells treated with cycloheximide only; *middle panel*, dead cells after treatment with TNF + cycloheximide; *bottom panel*, cells treated with TNF + cycloheximide in the presence of 250 units/ml [illegible]

Purification of the TNF-binding Protein

Concentration of the Crude Urinary Proteins (CUP)—Urine from healthy male donors was processed in pools of 300 liters. The urine was filtered on a Millipore HVLP membrane (pore size, 0.5 μm) using a Pellicon cassette system. The filtrate was concentrated by tangential ultrafiltration to a final volume of 750 ml, with the aid of a PTGC Millipore membrane having a molecular weight cut off at 10,000. The concentrate was dialyzed against PBS containing 0.02% NaN_3 and 1 mM benzamidine (Sigma), divided into portions, and frozen.

Chromatography on CM-Sepharose—A CM-Sepharose (Pharmacia, Uppsala, Sweden) cation exchange column (2.7 × 10 cm) was prewashed with 1 M NaCl, 10 mM citric acid, pH 5.0, containing 0.02% NaN_3 (buffer C) and equilibrated with 10 mM citric acid (pH 5) containing 0.02% NaN_3 (buffer A). The concentrate of urinary proteins was dialyzed against buffer A and centrifuged for 15 min at 8000 × *g*. The supernatant was applied at 4 °C on the column at a flow rate of 2 ml/min. The column was washed with 1500 ml of buffer A and eluted with 250 ml of a solution containing 200 mM NaCl, 10 mM citric acid (pH 5.0), and 0.02% NaN_3 (buffer B). A second step of elution was performed with 150 ml of buffer C. Fractions of 50 ml were collected and tested for biological activity, and their protein concentration was determined.

Cation Exchange HPLC—The active fractions eluted from the CM-Sepharose column were pooled, dialyzed against buffer A, and applied on a Mono S HR 5 × 50-mm column (Pharmacia). The column was washed at a flow rate of 0.5 ml/min until all unbound proteins were removed. The bound proteins were eluted with a linear NaCl gradient (0–350 mM) in buffer A. The gradient was run for 40 min at a flow rate of 0.5 ml/min. The column was then washed for 10 min in buffer D (350 mM NaCl in buffer A), and further with buffer C. Fractions of 0.5 ml were collected and examined for a protective effect against TNF cytotoxicity, and their protein concentration was determined.

Anion Exchange HPLC—A fast protein liquid chromatography Mono Q HR 5 × 50-mm anion exchange column (Pharmacia) was equilibrated with 5 mM sodium borate (pH 9.0) containing 0.02% NaN_3 (buffer E). The active fractions eluted from the Mono S column were pooled, dialyzed against buffer E, and loaded on the Mono Q column. The column was washed with buffer E until all unbound proteins were removed. The bound proteins were eluted at a flow rate of 0.5 ml/min, with a 30-min linear gradient from 0 to 60 mM NaCl in buffer E followed by a 20-min linear gradient from [illegible]

buffer E. Fractions of 0.5 ml were collected and examined as above.

Reversed Phase HPLC—The reversed phase HPLC column aquapore RP300 (4.6 × 30 mm, Brownlee Labs), was prewashed with 0.3% aqueous trifluoroacetic acid (buffer F). The active fractions from the Mono Q column were pooled and loaded on the column. The column was washed with buffer E at a flow rate of 0.5 ml/min until all unbound material was removed; it was then eluted at a flow rate of 0.5 ml/min with a 0–20% linear gradient of acetonitrile in buffer F for 5 min followed by a 20–50% linear gradient of acetonitrile in buffer F for 60 min, and finally a 50–80% linear gradient of acetonitrile in buffer F for 5 min. The column was then washed with 80% acetonitrile in buffer F for 15 min. Fractions of 0.5 ml were collected and assayed as indicated.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% acrylamide gels was performed by the method of Laemmli (12) using the Bio-Rad Minigel device (thickness of gel; 0.5 mm). Proteins in the gel were visualized by silver staining (13).

N-terminal Sequence Analysis—Samples of the purified TBP were subjected to N-terminal sequence analysis on a pulsed liquid gas phase protein microsequencer (Model 475A Applied Biosystems Inc., Foster City, CA).

Protein Determination—Protein concentrations were determined by the fluorescamine method (14), using crystalline bovine serum albumin as a standard.

RESULTS

Effects of Unfractionated Preparations of the Urinary Proteins on TNF Function—Unfractionated preparations of the proteins of human urine had a marked, concentration-dependent, protective effect against the cytotoxicity of TNF (Fig. 2*A*, and see also Fig. 1).

To explore the mechanisms which underly this protection, we examined the effect of the proteins on the binding of TNF to its receptors. This test was carried out in several different ways: (*a*) pretreating the cells with the urine proteins at 4 °C, prior to the quantitation of TNF binding, to find out if these proteins block the TNF receptors; (*b*) pretreating the cells with the proteins at 37 °C, to see if there are components in the urine which down-regulate the TNF receptors; (*c*) supplying the proteins, simultaneously with TNF, directly to the TNF binding assay mixture, to find out if constituents of the urine interfere with the binding of TNF by an effect on TNF itself.

Pretreatment of human foreskin fibroblast at 4 °C with the crude urinary proteins (CUP) had no effect on subsequent binding of TNF in the absence of the CUP (Fig. 3, *left, top panel*, 4 °C). However, after treatment with CUP at 37 °C, the cells exhibited a marked decrease in ability to bind TNF. This induced effect was rapid and transient, reaching a maximum within an hour of application of the proteins and then gradually decreasing (Fig. 3, *left, top panel*). A similar induced decrease in TNF binding has been reported in certain cells following treatment with IL-1 (15). Indeed, treating the human fibroblasts with IL-1, resulted in a decrease in TNF binding displaying kinetics similar to the kinetics of the effect induced by CUP (Fig. 3, *left, middle panel*). Murine A9 cells treated with CUP did not exhibit any such induced decrease in binding of TNF (not shown).

In addition, the CUP had a marked inhibitory effect on TNF binding when applied directly into the TNF binding

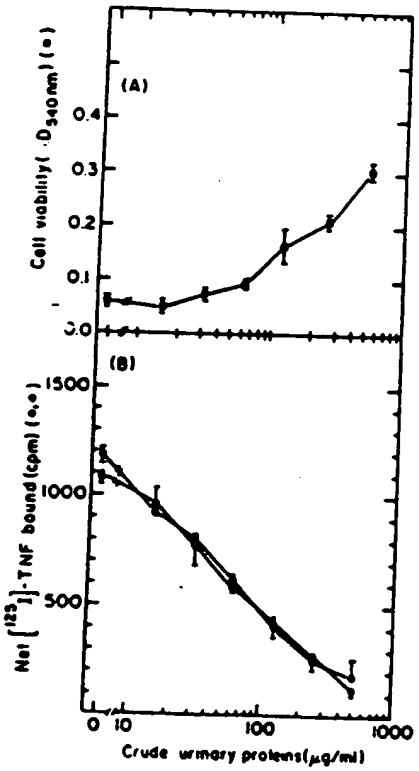

FIG. 2. Concentration dependence of the effects of unfractionated urinary protein (CUP) on TNF cytotoxicity (*A*) and TNF binding to cells (*B*). Viability of A9 cells following challenge with TNF in the presence of CUP at various concentrations (*A*) is demonstrated by the extent of uptake of neutral red dye by the cells ($OD_{540\ nm}$). Effect of the proteins on the binding of ^{125}I-TNF to FS11

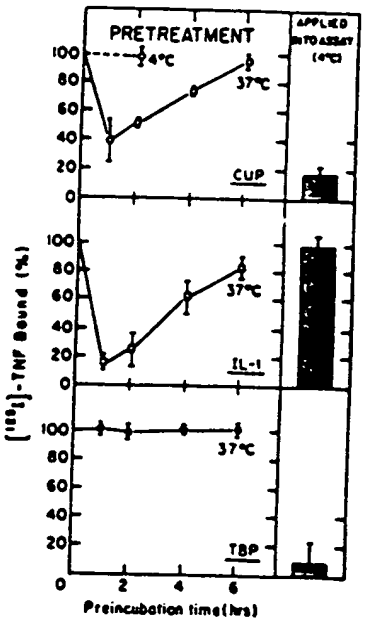

FIG. 3. Influence of the crude urinary proteins, IL-1α and pure TNF-binding protein on the binding of ^{125}I-TNF to FS11 cells. *Left*, pretreatment with the proteins; *right*, application of the proteins together with ^{125}I-TNF to the binding assay; *top*, effects of the crude urinary proteins (CUP) at 510 μg/ml; *middle*, effects of IL-1α at either 4 fg/ml (10 units/ml, *left*) or 4 μg/ml (*right*); *bottom*, effects of the purified TNF-binding protein (TBP) at 10 ng/ml. In the experiment presented in the *left panels*, cells were preincubated for the indicated duration either at 37 °C with the CUP (○), IL-1 (□), or TBP (●) or at 4 °C with CUP (◇). The proteins were then removed and the binding of ^{125}I-TNF to the cells was quantitated as described under "Materials and Methods." In the experiment presented in the *right panels*, CUP, IL-1, and TBP were applied directly into the TNF-

assay (Fig. 3, *right, top panel*). The effect occurred also at 4 °C and could not be increased further by preincubating TNF with the CUP at 37 °C (Fig. 2*B*). It could be observed in all cells examined, including the human foreskin fibroblasts (Fig. 2) and murine A9 cells (Table I). The CUP also interfered with the binding of TNF-β (lymphotoxin) and IL-1 to cells, when applied simultaneously with the cytokines (Table I).

Several trivial causes for the effects of urinary proteins on the activity of TNF seemed to be excluded by the above observations. The presence of TNF itself in the urine would have resulted in interference by the urinary proteins with the binding of radiolabeled TNF and, in certain cells, also in induction of resistance to TNF toxicity (16). However this possibility seemed to be excluded by the fact that the urinary proteins did not interfere with the binding of TNF when applied to cells in the cold, prior to the application of TNF. Were TNF present in a free form in the urine, it would be expected to bind to the TNF receptors in such a pretreatment and thus block the binding of subsequently applied radiolabeled TNF. The presence of biologically active TNF in the CUP seemed to be excluded also by the fact that these preparations did not have any toxic effect on cells, not even when applied in the presence of cycloheximide, which sensitizes cells to TNF toxicity (data not shown).

IL-1 has also been shown to induce, in certain cells, resistance to the toxicity of TNF, as well as a decrease in TNF binding (15, 17). However, unlike the urinary proteins, IL-1 does not interfere with TNF binding when applied to cells simultaneously with TNF, in the cold (Fig. 3, *right, middle panel*; see also Ref. 15) and, therefore, even if present in the urine, cannot account for that effect of CUP.

We also considered the possibility that the decrease in TNF binding and activity was due to degradation of TNF by some proteases known to be present in the urine. The fact that incubation of TNF with the urine proteins at 37 °C for 2 h prior to their application to the cells did not enhance the interference of the CUP with TNF binding (Fig. 2*B*), testifies that neither proteolytic degradation nor any other enzymic modification of TNF is involved in this effect. Furthermore, analysis by SDS-PAGE of the molecular size of TNF after its incubation with the urinary proteins revealed no signs of such degradation (not shown). Several agents known to block protease activity, *N*-ethylmaleimide, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, benzamidine, iodoacetamide, phenylmethanesulfonyl fluoride (all tested at a concentration of 1 mM), leupeptin (at 1 μg/ml), and aprotinin (at 0.5 unit/ml), did not interfere with the inhibition of TNF binding by the CUP (data not shown).

Involvement of antibodies in the effect of CUP seemed unlikely since, normally, urine does not contain antibodies. Furthermore, the molecular size of the proteins which mediate the effect on TNF activity, as estimated by size exclusion chromatography (see below), is clearly lower than that of immunoglobulins.

Purification and Initial Characterization of the Urinary TNF-binding Protein—A bioassay for the urinary protein(s) which mediate a protective effect against TNF cytotoxicity was established (see "Materials and Methods"); it then was used for the detection of these protein(s) throughout various fractionation steps.

Several chromatographic approaches for purification of the protein(s) were attempted. In size exclusion chromatography (on an Ultrogel AcA44 column equilibrated with phosphate-buffered saline) only poor resolution between the protein(s) which interfere with TNF activity and other urinary proteins could be observed. Under these conditions, the activity fractionated together with the majority of the protein mass, peaking at an apparent molecular size of about 50,000–70,000. On the other hand, in isoelectric focusing, some enrichment of the proteins could be obtained. Consistent with prior observations, much of the protein mass of urine was found to be rather acidic, with an apparent isoelectric point lower than 5.0. Yet the isoelectric point of the protein(s) which protect cells from TNF toxicity was found to be close to 6.0 (data not shown). Fractionation by isoelectrofocusing for initial enrichment of the protein seemed impractical since only limited amounts of protein can be applied at a time. However, the pattern of isoelectric points revealed by that procedure indicated that enrichment of the protective protein on the basis of its charge properties should be possible.

As a first step, the urinary proteins were fractionated at pH 5.0 on a carboxymethyl-Sepharose column. Consistent with their acidity, most of the proteins did not bind to the resin under those conditions. However, the inhibitor of TNF activity was bound effectively to the resin at that step (about 80% of the applied activity), and most of it could be eluted from the column, together with about 1% of the initially applied protein, by increasing the ionic strength by 0.2 M NaCl. Increasing the ionic strength further (1 M NaCl) did not result in elution of any additional activity.

In the second purification step, the proteins were fractionated on a cation exchange HPLC column. The active protein was eluted at about 180–220 mM NaCl (Fig. 4). Peak fractions

TABLE I

Interference of the urinary proteins with the binding of various cytokines to cells

The various cytokines, radiolabeled with ^{125}I, were applied to cells either alone, or together with the crude urinary proteins (CUP, 510 μg/ml) or the pure TNF-binding protein (TBP, 10 ng/ml). Specific binding was determined as described under "Materials and Methods." Values are given as percent of the binding of each cytokine in the absence of the inhibitory protein, which for rhuTNF-α was 2160 cpm (±160); for rhuTNF-β, 1210 cpm (±102); for rhuIL-1α, 1019 cpm (±69); and for rmuTNF-α, 13,543 cpm (±188).

	Binding to human FS11 cells			rmuTNF-α binding to A9 cells
	rhuTNF-α	rhuTNF-β	rIL-1α	
	%	%	%	%
Untreated cells	100	100	100	100
CUP (7 units/ml)	11 ± 5	35 ± 5	59 ± 11	22 ± 1

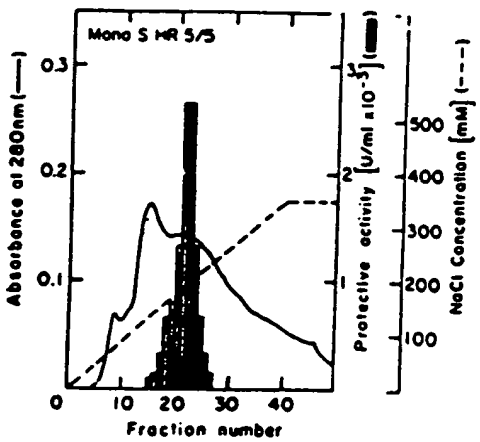

FIG. 4. Mono S cation exchange HPLC of TBP. The column was equilibrated with 10 mM citric acid (pH 5), 0.02% NaN_3. Urinary proteins, enriched for TBP activity by fractionation on CM-Sepharose, were applied and then eluted with NaCl gradient (- - -). The

were pooled and subjected to further purification on an anion exchange HPLC column, from which the TNF inhibitory activity was eluted at a salt concentration of about 40 mM (Fig. 5).

The final fractionation step was on an Aquapore RP 300 reversed phase HPLC column. Proteins were eluted from the column by applying a gradient of acetonitrile. The active protein was found to elute as a distinct protein peak, at about 27% acetonitrile (Fig. 6).

Analysis by SDS-PAGE, revealed that the factor was purified at that step to homogeneity; it was identified as a protein with an apparent molecular weight of about 27,000 (*inset* in Fig. 6). The molecular size was independent of whether or not the analysis was performed in the presence of a reducing agent (β-mercaptoethanol). Homogeneity of the purified protein was further confirmed by N-terminal microsequence analysis. A single sequence, Asp-Ser-Val-Cys-Pro-, was obtained in the analysis at a high yield (initial yield, 67%).

Both from the low amounts of the protein, recovered in the purification, and from comparison with the protein pattern in the crude preparation, as revealed by SDS-PAGE, it is clear that the factor is a very minor constituent of urine. In the urine preparation, used for the fractionation whose results are presented in Table II, the protein constituted 10^{-3}–10^{-4}% of the total protein. Specific activity of the purified protein was about 50,000-fold higher than that of the CUP.

Like the crude preparations of the urinary proteins, the purified protein interfered with the binding of TNF-α (of both human and mouse origin), as well as with huTNF-β when applied to cells simultaneously with these cytokines (Table I and Fig. 3, *right, bottom panel*). However it did not interfere, to any measurable extent, with the binding of IL-1 to its receptor (Table I) nor did it induce in cells, pretreated with the protein, a decrease in ability to bind TNF (Fig. 3, *left, bottom panel*). The latter two effects of crude preparations of the urinary proteins therefore seem to be mediated by some other constituents of the urine.

The binding properties of the urinary protein were explored using radiolabeled preparations of the purified protein. As shown in Table III, the labeled protein was found to bind to immobilized TNF-α and, to a much lesser extent, to TNF-β. This binding could be competed by TNF-α and TNF-β and by excess of the unlabeled urinary protein. IL-1 (as well as IFN-γ) did not bind the urinary protein, nor did it compete for the binding of the protein to TNF.

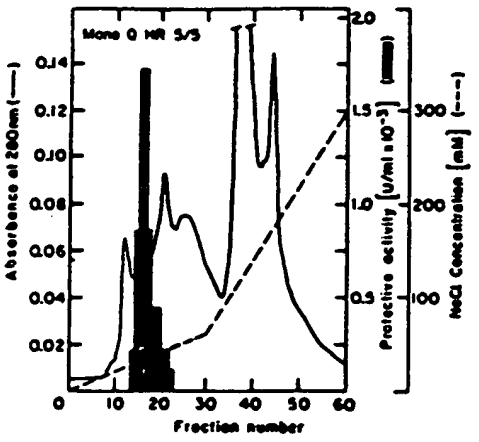

FIG. 5. **Mono Q anion exchange HPLC of TBP.** Active fractions eluting from the Mono S column were made up to 5 mM sodium

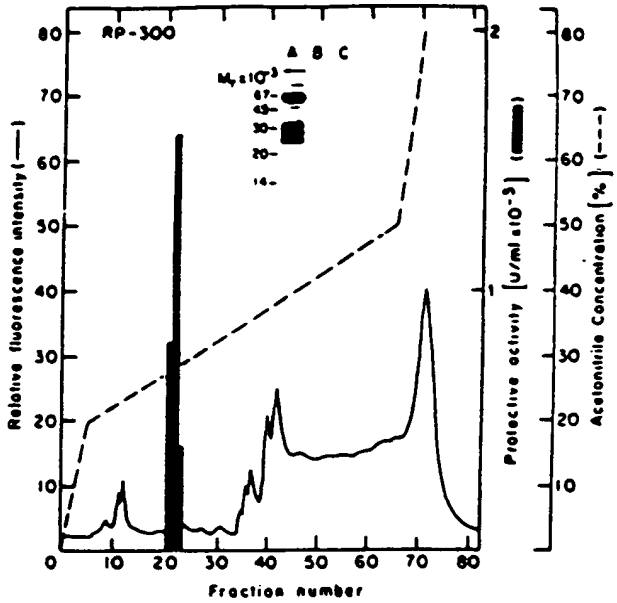

FIG. 6. **Reversed phase HPLC of TBP.** The proteins enriched for TBP activity on the Mono Q column were applied to an Aquapore RP300 column. Elution was performed with a gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid (- - -). Fractions were examined for bioactivity (▮), and protein (——) content. Shown in *inset* is SDS-PAGE analysis of proteins in the absence of reducing agents (*A, B*), or in the presence of 15% β-mercaptoethanol (*C*). The pattern of proteins in the preparation of unfractionated urinary proteins prior to chromatography on CM-Sepharose (5 μg, *lane A*), is compared to the protein in fraction 21 of the elution from the HPLC RP300 column (0.1 μg, *lanes B* and *C*), where only a single polypeptide band can be discerned. (The faint high molecular weight bands in *C* could be observed also in the absence of any added protein and seem to reflect the presence of some contaminants in the β-mercaptoethanol.) By comparing to the migration of molecular weight markers (Pharmacia, shown in the *left*), the molecular weight of the purified TBP was estimated as about 27,000.

TABLE II

Purification of the TNF-binding protein

Purification step	Protein	Protective activity	Specific activity	Purification	
				Per step	Total
	mg	*units*	%	*units/mg*	*-fold*
Starting material (CUP)	7.1×10^3	90,100	100	13	
Carboxymethyl-Sepharose	8.9×10^1	54,400	60	610	48	48
Mono S	1.7×10^1	20,000	22	1,120	1.8	88
Mono Q	4.8×10^{-1}	13,000	14	29,100	24.3	2,100
HPLC RP 300	1.1×10^{-2}	7,000	8	600,000	23.8	54,000

DISCUSSION

A protein present in the human urine in minute amounts is shown in this study to interfere with the function of TNF by blocking the binding of TNF to its receptors. In the crude state, the urinary proteins suppress the binding of TNF to cells by effects on both the cell and the TNF molecules; they also interfere with the interaction of cells with IL-1-cytokines

TABLE III

Binding of the TNF-binding protein to cytokines and the effect of competitive proteins

Binding of purified, radiolabeled TBP to the cytokines listed in the left column and cross-competition with the cytokines indicated or with excess unlabeled TBP (last column) was determined in a solid phase assay as described under "Materials and Methods." The concentrations of the proteins applied for competition for the binding to TBP were as follows: TNF-α, 5 μg/ml; TNF-β, 1 μg/ml; IL-1α, 4 μg/ml; IFN-γ, 1 μg/ml; TBP, 80 μg/ml.

Cytokine examined for binding to TBP		Proteins applied for competition for TBP binding				
		TNF-α	TNF-β	IL-1α	IFN-γ	TBP
		cpm [^{125}I]TBP bound				
TNFα	17,820 (±130)	400 (±10)	14,340 (±900)	18,520 (±450)	17,810 (±700)	790 (±20)
TNFβ	450 (±40)	130 (±10)	240 (±30)	480 (±50)	440 (±20)	110 (±10)
IL-1α	140 (±40)	130 (±30)	105 (±10)	100 (±10)	130 (±30)	130 (±30)
IFNγ	110 (±10)	100 (±10)	105 (±10)	90 (±10)	120 (±20)	120 (±10)

crude urine against TNF toxicity, seems to function only by affecting the TNF molecules; it showed no direct effect on cells and was unable to interfere with the binding of IL-1 to its receptors.

The purified protein acts by binding the cytokine and thus competing for it with the TNF receptors. Although it does not bind IL-1, it does bind TNF-α and, with much lower effectivity, also TNF-β (lymphotoxin). The specific nature of its interaction is further demonstrated by the inability of the protein to bind another cytokine-IFN-γ. Even though TNF-α and TNF-β share only partial structural homology (18), they compete for binding to the same cell surface receptor (19). The fact that they both bind to the urinary protein raises the possibility that this protein associates with that part of the cytokine molecule which is recognized by the receptor.

By its inability to interfere with the binding of IL-1 to cells, the TNF-binding protein (TBP) can be distinguished from uromodulin, a major glycoprotein of the urine, of greater molecular size (85,000), which was reported to have a high binding affinity to both IL-1 and TNF and to interfere with the function of IL-1, although not with that of TNF (5–7). The TBP is also clearly distinct from another antagonist to IL-1, shown recently to be present in urine, which interferes with IL-1 binding apparently by binding competitively to the IL-1 receptor, but it seems unable to block the function of TNF (4, 20, 21).

The presence of the above antagonists in urine probably accounts for the inhibitory effect of CUP on IL-1 binding. At the same time, the induced decrease in the ability of FS11 cells to bind TNF following treatment with CUP appears to be very similar to an effect of IL-1 itself. IL-1 induces a decrease in binding of TNF, which apparently reflects a decrease in expression of the TNF receptors. In FS11 fibroblasts, although not in some other cells (15), this decrease is transient and its kinetics resembles the one seen with urinary proteins. Some evidence for the presence of IL-1 in the urine has been reported (4, 22). Whether indeed IL-1, if present in the unfractionated preparations of the urine, can mediate the effect in spite of its coexistence with proteins which block its activity, or whether it is some other constituent(s) of the urine which functions in our test system similarly to IL-1, remains to be determined.

The protein whose purification is described in this study is present in the urine in minute amounts, constituting about 10^{-3}–10^{-4}% of the total protein mass. It would not have been detected save for the fact that it is very active. Indeed, it can be calculated that a unit of protective activity (the activity resulting in a 2-fold increase in the number of cells which remain viable after challenge with TNF) is mediated by the

The physiological role of the protein remains to be elucidated. It is tempting to speculate that, just as in our *in vitro* experimental system, this protein [illegible] *vivo* as an antagonist to TNF. However, to test this hypothesis it will be necessary first to determine how this protein is formed and what results from its interaction with TNF under *in vivo* conditions. At present, one cannot rule out that this protein has a completely different *in vivo* role, perhaps even a converse one; it might prolong the exposure of the organism to endogenously produced TNF by binding the cytokine and then releasing it slowly in an active form.

There is some evidence both for the existence of mechanisms whereby the organism can protect itself from the potentially destructive effects of TNF and for enhanced expression of these mechanisms following exposure to TNF. Several studies have shown that vulnerability to certain deleterious effects of TNF and of bacterial components which can induce TNF production is markedly reduced following exposure of the organism to TNF itself or to IL-1 (23–26). If the protein described in the present study can indeed contribute to such protection, it is very likely that useful applications for it will be found, specifically as a therapeutic agent in those pathological situations where TNF can have detrimental effects.

Acknowledgments—We thank Prof. M. Revel and Dr. E. Geller for encouragement and support; Dr. D. Novick for useful advice; Dr. C. Serafini, of the Cesare Serono Research Institute, Rome, Italy, for preparing the crude urinary proteins; Dr. H. Holtmann for comments on the manuscript; Dr. G. Adolf of the Boehringer Institute, Vienna, Austria, for gifts of rhuTNF-α, rhuTNF-β, and rmuTNF-α; Drs. A. Stern and P. T. Lomedico of the Roche Research Center, Nutley, NJ, for the gift of human rIL-1α. Our thanks are also due to Adela Dibeman and Mordechai Gabai for technical assistance and to N. Tal for performing the microsequencing.

Addendum—Recently, we have determined the sequence of the 18 N-terminal amino acids of the TBP. A search through the NBRF protein data bank, release 18, failed to reveal significant homology to any of the other known protein sequences. In the 2nd International Conference on TNF and Related Cytokines held January 15–20, this year, L. Olsson from the University of Lund, Sweden, also reported on the purification of a urinary protein which binds TNF. Based on comparison of N-terminal amino acid sequences and of chromatographic properties, this protein appears identical to the one whose purification is described in the present study.[1]

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Appendix L

LYMPHOKINE RESEARCH
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DA34

Mechanisms Which Take Part in Regulation of the Response to Tumor Necrosis Factor

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In view of increasing evidence for a causative role of TNF in the pathogenesis of certain diseases (1,2), there is particular interest in elucidation of the mechanisms by which the function of this cytokine is controlled. The studies described below provide initial information on two different reflections of those mechanisms. One, concerns a mechanism which may contribute to a marked sensitization to some deleterious effects of TNF, observed in animals which were afflicted with pathogens (3-6). The other was initiated in an attempt to shed light on the mechanisms by which a decrease in responsiveness to TNF, such as observed following preexposure to a low dose of TNF, may occur (7,8).

Pathogen-mediated Enhancement of the Prostaglandin Inducing Effect of TNF

Vulnerability of experimental animals to the lethal effect of injected TNF was found, in a number of studies, to increase by certain pathogenic agents, like bacteria, the malaria parasites and tumors (3-6). Other studies (e.g. cf. 9) suggested that some deleterious effects of TNF are precipitated by prostaglandins, produced in response to it. A possible mechanistic link between the above observations was indicated in a study in which we examined the effect of TNF on cells after their infection with Chlamydia trachomatis. As shown in Table 1 growth of the Chlamydia in HEp-2 cells is significantly inhibited by rTNF (see also 10). An even further inhibition is observed when treating the cells with TNF together with IFN-γ, at concentrations of IFN at which IFN alone has only little effect (10,11). It has been reported that the growth of chlamydiae is inhibited also by prostaglandins (12). We therefore examined the production of prostaglandins in infected cells which were treated with TNF. Similarly to various other cells (13,14) uninfected HEp-2 cells respond to TNF in some production of PGE_2, though to only a little extent. Infection with Chlamydia, which by itself results in only little induction of PGE_2 potentiates the TNF effect (Table 1). Maximal induction of PGE_2 is observed at concentrations of TNF which effectively inhibit the chlamydial growth. Treating the cells with TNF and IFN-γ together, which practically abolishes the growth of chlamydia results in a similar, pronounced induction of PGE_2.

TABLE 1:

Effect of TNF-α and Chlamydia on the Production of PGE_2 in the HEP-2 Cells

	Chlamydial yield (IFU/ml)	PGE_2 (pg/ml)
Control	-	54
Chlamydia	$(5.1 \pm 0.8) \times 10^6$	510
TNF	-	405
Chlamydia + TNF	220 ± 92	42000

Where indicated, cells were infected with chlamydia trachomatis (L2/434/Bu) at a multiplicity of infections of 1 and, following adsorption of the bacteria, treated with rTNF-α at 500 ng/ml. Chlamydial yield and the amounts of PGE_2 in the growth medium were determined at 48 h after infection.

Such a potentiative effect of a pathogen of an effect of TNF which can be inhibitory to its growth, provides an interesting demonstration for a mechanism by which a protective function of TNF can be adjusted to the need. Yet, when occurring in excess, the augmented production of PGE_2 at the time of exposure to TNF may just as well contribute to the precipitation of the deleterious effects of this cytokine.

Isolation from the Human Urine of a TNF Binding Protein with an Inhibitory Effect on TNF Activity

Trying to identify regulatory molecules which may contribute to the decrease in responsiveness to TNF (7,8) we followed prior studies which indicated that the function of IL-1 can be suppressed by certain secreted inhibitors. Human urine has been shown to contain such inhibitors of IL-1 activity (15-17). We therefore examined whether the urine contains also components which can affect cell response to TNF. As demonstrated in Fig. 1, concentrated preparations of the urinary proteins indeed suppressed effectively the cytocidal activity of TNF (18,19). A similar observation has been reported also by others (20,21).

Examining further the effect of these proteins on the binding of radiolabelled TNF to cells, we found that in certain cells unfractionated preparations of the proteins of human urine induce, in a temperature dependent way, a rapid, though transient decrease in the expression of the TNF receptors; quite similarly to an effect which IL-1 can have in those cells (23 and see also Fig. 2). The proteins of urine did not interfere with the binding of TNF when applied to cells in the cold and then removed, prior to the application of radiolabelled TNF, ruling out that their effect reflects the function of a protein which binds to the TNF receptors competitively with TNF. However, distinctly from the way IL-1 can affect TNF binding, the urinary proteins did inhibit the binding of TNF to cells, even in the cold, when applied to them simultaneously with it (Fig. 2).

Applying a series of chromatographic steps we isolated from the urine the

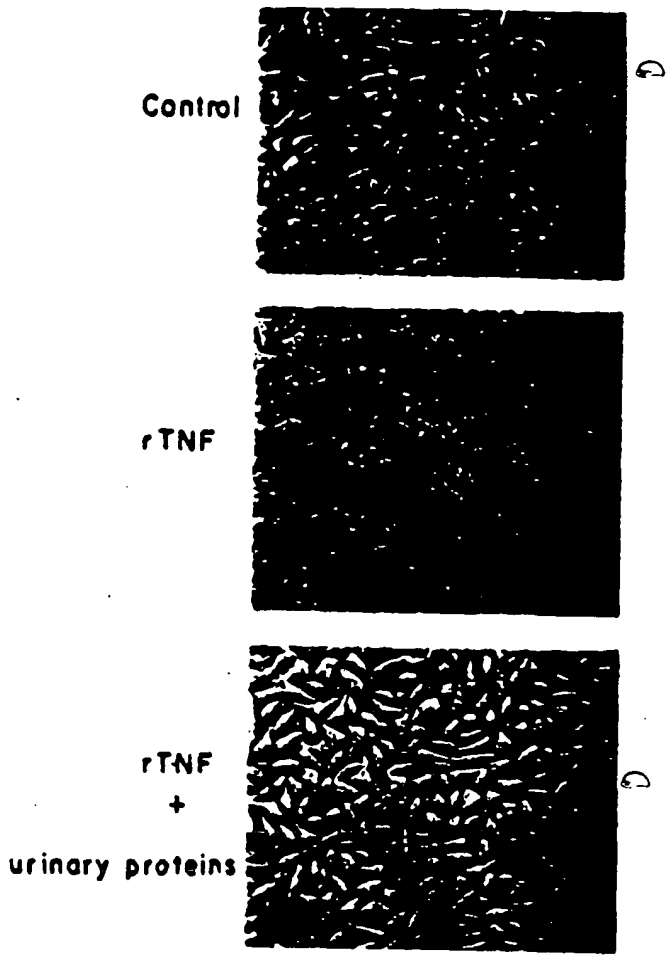

Figure 1. Protective effect of the human urinary proteins against the cytocidal effect of TNF. Top panel: Control. A9 cells treated for 14h with cyclohexamide only (CHI, 50µg/ml). Middle panel: dead cells after treatment with rTNF-α (5U/ml) + CHI. Bottom panel: cells treated with TNF+CHI in the presence of a preparation of the urinary proteins.

protein which exerts the latter effect. It turned to be a very minor constituent of the normal urine (about 10^{-3}% of the proteins). Unlike the crude preparations, the purified protein did not induce a decrease in the expression of TNF receptors in tissue cultured cells. It was found to affect TNF by binding to it. It also binds TNF-β (LT), though at lower effectivity but not IL-1 nor IFN-γ. The protein, whose apparent molecular weight in SDS PAGE analysis was found about 30 KDa, appears, by its amino acid sequence, to be distinct from all previously isolated proteins (19).

The cellular source of this urine-derived TNF-binding protein remains to be elucidated. A likely possibility, in view of the specific nature of its binding activity, is that this protein is a shed fragment of the cell surface TNF receptor. Indeed the urine has been shown to contain a soluble form of the IL-2 receptor, which in potential may impede the function of this cytokine (23). It is tempting to speculate that the formation of the soluble TNF binding protein constitutes a way of restraining the function of TNF. Whether this indeed is its physiological role or not, the possible use of the protein in therapy for blocking the function of TNF, at times when it contributes to pathogenicity, seems worthy of exploring.

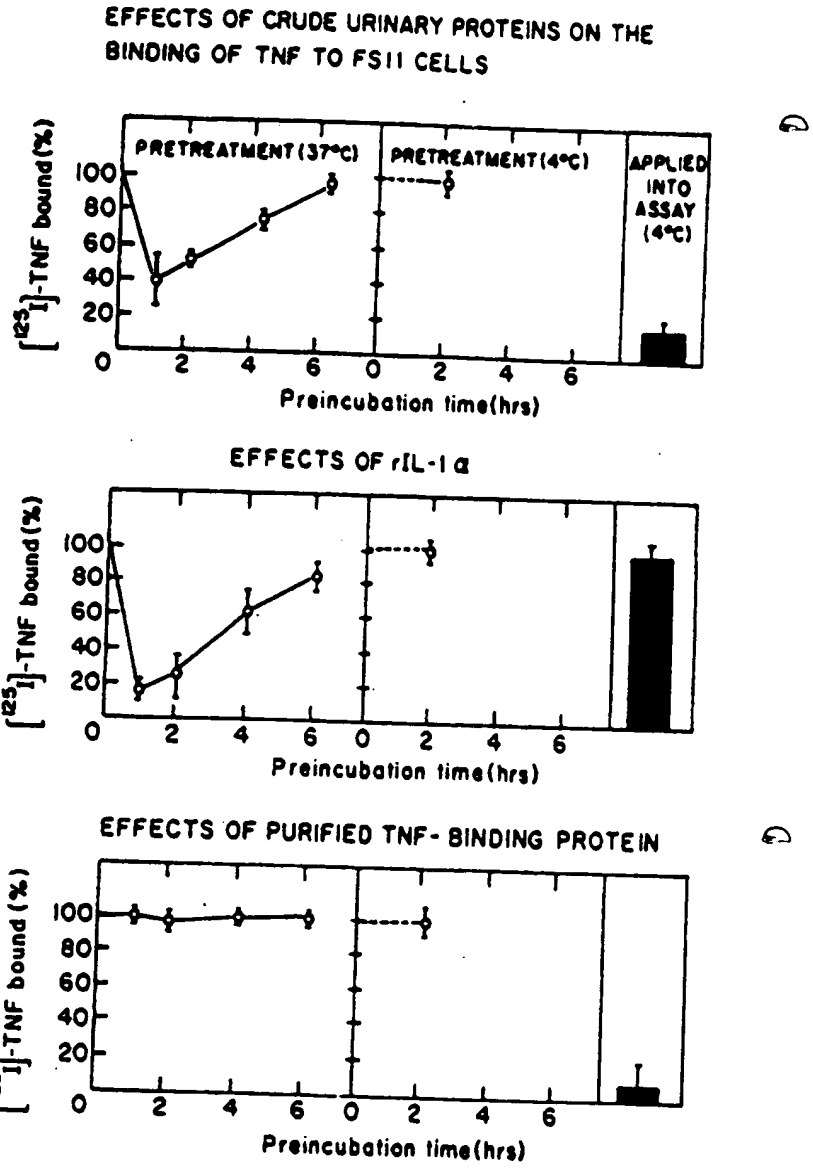

Figure 2. Influence of the crude urinary proteins, IL-1α and pure TNF binding protein on the binding of ^{125}I-TNF to FS11 cells. Left and middle panels - Pretreatment with the proteins at 37°C and 4°C respectively. Right - Application of the proteins together with ^{125}I-TNF to the binding assay. Top: Effects of the crude urinary proteins (CUP) at 510 μg/ml. Middle: Effects of IL-1α at either 4 pg/ml (10 u/ml-left and middle) or 4 μg/ml (right). Bottom: Effects of the purified TNF binding protein (TBP) at 10 ng/ml. In the experiments presented in the left and middle panels cells were preincubated for the indicated duration either at 37°C or at 4°C with the CUP, IL-1 or TBP. The proteins were then removed and the binding of ^{125}I TNF to the cells was quantitated. In the experiment presented in the right panels CUP, IL-1 and TBP were applied directly into the TNF-binding assay mixture (assay performed at 4°C). Specific binding of TNF in the absence of CUP, TBP or IL-1 (100%) was 1370 cpm (±107) (19).

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Comparison of sequences provided in D18.1 (Fig. 4) and D18.2 (Fig. 1D)

↓: Deletion or substitution in nucleic acid sequence
↑: Deletion or substitution in amino acid sequence

```
↓↓↓ ↓↓↓     ↓↓↓ ↓
Gly Val Ile Gly Leu Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile
GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC
TAT CGA ATT --- CCG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC
Tyr Arg Ile     Pro Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile
 ↑   ↑       ↑   ↑

                                                                    ↓
His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro
CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG
CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TTC TTG TAC AAT GAC TGT CCA GGC CCG
His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys Gly Thr Phe Leu Tyr Asn Asp Cys Pro Gly Pro
                                                                    ↑

                                                          ↓         ↓
Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu
GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC
GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC AC- GCT TCA G-A AAC CAC CTC AGA CAC TGC CTC
Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe  Thr Leu Gln  Lys Pro Pro Gln Thr Leu Pro Gln
                                                          ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑

                                        ↓   ↓     ↓   ↓   ↓
Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys
AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT
AGC TGC TCC AAA TGC CGA AAG GAA -TG G-T CAG -TG -AG A-C TCT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT
  Leu  Leu Gln Met Pro Lys Gly Met  Val  Ser  Glu   Thr  Leu Leu Ala Gln Trp Thr Gly Thr Pro Cys Val
   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑    ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑
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Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu
GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC
GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC
 Ala Ala Gly Arg Thr Ser Thr Gly Ile Ile Gly Val Lys Thr Phe Ser Ser Ala Ser Ile Ala Ala Ser Ala Ser
  ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑

                                                                                  ↓  ↓↓
Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg
AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA
AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GC- G-A TTC TTT CTA AGA
 Met Gly Pro Cys Thr Ser Pro Ala Arg Arg Asn Arg Thr Pro Cys Ala Pro Ala Met  Arg   Phe Phe Leu Arg
  ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑   ↑

                                                                              ↓
Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu
GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG
GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTC TGC CTA CCC CAG ATT GAG
Glu Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu

          ↓                                          ↓ ↓       ↓↓↓ ↓
Asn Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu
AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA GTG CTG TTG CCC CTG GTC ATT TTC TTT GGT CTT TGC CTT TTA
AAT GTT AAC GGC ACT GAG GAC TCA GGC ACC ACA GTC GTG TTG AAA ATG GTC ATT TTC TTT GGT CTT TGC CTT TTA
Asn Val Lys Gly Thr Glu Asp Ser Gly Thr Thr Val Val Leu Lys Met Val Ile Phe Phe Gly Leu Cys Leu Leu
                                                  ↑       ↑   ↑

Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG
TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG
Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
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Appendix N

Proc. Natl. Acad. Sci. USA
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Biochemistry

Three human transforming genes are related to the viral *ras* oncogenes

(human tumor cells/molecular cloning/gene families)

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ABSTRACT Three distinct transforming genes present in human tumor cell lines are all related to the viral oncogenes of Harvey and Kirsten murine sarcoma viruses, designated v-H-*ras* and v-K-*ras*, respectively. The transforming gene of a bladder carcinoma cell line has been shown to be a human homolog to v-H-*ras* [Parada, L. F., Tabin, C. J., Shih, C. & Weinberg, R. A. (1982) *Nature (London)* 297, 474–478; Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. & Barbacid, M. (1982) *Nature (London)* 298, 343–347]. The transforming gene common to one colon (SK-CO-1) and two lung carcinoma (SK-LU-1 and Calu-1) cell lines is the same human homolog of v-K-*ras* as is the transforming gene previously identified in a lung carcinoma cell line Lx-1 [Der, C. J., Krontiris, T. G. & Cooper, G. M. (1982) *Proc. Natl. Acad. Sci. USA* 79, 3637–3640]. The transforming gene of SK-N-SH neuroblastoma cells is weakly homologous to both v-H-*ras* and v-K-*ras*. NIH 3T3 cells transformed with the SK-N-SH transforming gene contain increased levels of a protein serologically and structurally related to the protein products of the v-H-*ras* and v-K-*ras* genes. Therefore, it represents a third member of the *ras* gene family, which we have called N-*ras*. Based on the homology with the v-*ras* genes, we have established the orientation of transcription and approximate coding regions of the cloned human K-*ras* and N-*ras* genes.

The progression of a cell from normalcy to malignancy may be due in part to the activation of transforming genes of cellular origin. The existence of cellular transforming genes has been demonstrated by the ability of genomic DNAs from certain tumors and cell lines to induce foci of transformed NIH 3T3 cells after DNA-mediated gene transfer. Transforming genes in rodent (1, 2) and human (3–9) tumor cells have been detected in this way. We have detected three distinct transforming genes in our study of 21 human tumor cell lines: one common to two lung carcinoma (SK-LU-1 and Calu-1) and colon carcinoma (SK-CO-1) cell lines, one in a bladder carcinoma (T24), and one in a neuroblastoma (SK-N-SH) cell line (9).

Several research groups have shown that certain transforming genes detected by transfer to NIH 3T3 cells are related to viral oncogenes. Der *et al.* (5), Parada *et al.* (10), and Santos *et al.* (11) have demonstrated that the transforming gene of T24 and EJ, two human bladder carcinoma cell lines that probably are derived from the same source (unpublished data), is the human homolog of v-H-*ras*, the oncogene of the Harvey sarcoma virus. Der *et al.* (5) have also shown that the transforming gene of Lx-1, a human lung carcinoma cell line, is a human homolog of v-K-*ras*, the oncogene of the Kirsten sarcoma virus. The genes that we have isolated from human tumor cell lines are related also to the viral oncogenes, designated v-*onc*. We demonstrate that the transforming gene common to Calu-1, SK-LU-1, and SK-CO-1, like the transforming gene in Lx-1 characterized by Der *et al.* (5), is a human homolog to v-K-*ras*. We also demonstrate that the transforming gene of SK-N-SH is related to both v-K-*ras* and v-H-*ras* and probably codes for an immunologically crossreactive and structurally related protein. Based on the homology with the v-*ras* genes, we have established the orientation of transcription and probable coding regions of these genes.

MATERIALS AND METHODS

Human Tissue and Tissue Culture Cell Lines. T24, Calu-1, SK-LU-1, SK-CO-1, and SK-N-SH are human tumor cell lines (9). HT14B is a NIH 3T3 cell line transformed by Harvey sarcoma virus unintegrated viral DNA. Other transformed cell lines are described in the text.

Preparation of DNA. DNA was prepared from tissue culture cells by $NaDodSO_4$/proteinase-K lysis and phenol/chloroform extraction as described (9). Plasmid and bacteriophage DNAs were prepared as described (12, 13).

Enzymes. Restriction endonucleases were purchased from New England BioLabs and Bethesda Research Laboratories and used according to suppliers' instructions. *E. coli* DNA polymerase I was purchased from Bethesda Research Laboratories, and pancreatic DNase I was from Worthington Biochemicals.

Southern Filter DNA Blot Hybridization. DNA samples were digested with restriction endonucleases and subjected to agarose gel electrophoresis and filter-blot transfer by the method of Southern (14). Filter-blotted DNAs were hybridized with a nick-translated ^{32}P-labeled DNA probe under two sets of conditions. Stringent hybridization conditions entailed hybridization in a mixture containing 6× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M Na citrate, pH 7.0), Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.2% bovine serum albumin), and denatured salmon sperm DNA (20 μg/ml) for 16 hr at 74°C (15), followed by sequential washing at 74°C with 2×, 1×, and 0.5× NaCl/Cit in 0.1% $NaDodSO_4$. Nonstringent hybridization conditions entailed hybridization in a mixture containing 30% (vol/vol) formamide, 6× NaCl/Cit, 2× Denhardt's solution, *E. coli* DNA (100 μg/ml), yeast RNA (200 μg/ml), 50 mM sodium phosphate (pH 7), and 10 mM EDTA at 37°C for 36 hr, followed by washing at 50°C in 6× NaCl/Cit/0.1% $NaDodSO_4$. Hybridized DNA was revealed by autoradiography.

Immunoprecipitation of Cellular Protein with Rat Anti-*ras* p21 Antiserum. NIH 3T3 normal and transformed cells were

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Abbreviations: NaCl/Cit, 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0; kbp, kilobase pairs.

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labeled in methionine-free medium containing 20 μCi (1 Ci = 3.7×10^{10} Bq) of [^{35}S]methionine per ml (New England Nuclear) for 18 hr. Labeled cells were lysed in phosphate-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 2 units of aprotinin per ml, and the lysates were sheared through a syringe and clarified at 100,000 × g for 45 min at 4°C. Clarified supernatants were preabsorbed with goat anti-rat IgG and *Staphylococcus aureus* protein A. Immunoprecipitation was performed with anti-v-H-*ras* p21 rat monoclonal antibody Y13-259 (16) (the gift of M. Furth and E. M. Scolnick) for 5 hr at 4°C, followed by addition of goat anti-rat IgG for 1 hr. Immune complexes were absorbed to protein A, and the protein A suspension was washed extensively in lysis buffer. Protein A pellets were boiled in NaDodSO$_4$ sample buffer and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis by the method of Blattler *et al.* (17). Radiolabeled proteins in gels were visualized by fluorography.

Molecular Clones. Clones of avian and mammalian v-*onc* genes are described in Table 1. λT24 and λP3 are clones from λL47.1 genomic libraries containing the transforming gene of the T24 cell line and the nontransforming homologous human sequences, respectively (25). pT24 is a pBR322 derivative with a 6.2-kilobase-pair (kbp) *Bam*HI insert bearing the T24 transforming gene. The SK-N-SH transforming gene is contained on a Charon 4A recombinant phage, λNPS-1-1-1 as described (26). (See also Fig. 4*B*.) λNPS-1-1-1 also contains portions of pBR322 and the *E. coli* tRNA *sup F* gene.

Cloning the Transforming Gene of Calu-1. An initial DNA clone of part of the transforming sequences of the Calu-1 transforming gene was obtained by using the strategy of Gusella *et al.* (27) as described by others (28, 29). DNA was prepared from NIH 3T3 secondary and tertiary transformants containing the transforming gene of Calu-1. Phage libraries were prepared from these DNAs in λ Charon 4A (30) by the method of Hohn and Murray (31) and screened for the presence of human sequence by the method of Benton and Davis (32); the probe was "BLUR8", a clone of the dispersed, repeated human "*Alu*" family sequences (33). One λ phage clone, λL2-34, was isolated this way. Unique sequence DNAs from this clone were then used as probes for isolating "contiguous" DNA from our λ Charon 4A libraries. More than 20 independent phages containing inserts with overlapping restriction endonuclease maps were isolated in this manner. A representative set of five overlapping phage isolates, together with a composite restriction endonuclease map of 26 kbp of cloned DNA, is shown in Fig. 4A. pLC3 is the 3.0-kbp *Eco*RI fragment of λL2-11 cloned into the *Eco*RI site of pBR322 (see Fig. 4*A*).

Table 1. Molecular clones of v-*onc* genes

v-*onc* designation	Virus of origin*	Molecular clone	Restriction fragments bearing v-*onc*	Ref. no.
fps†	PRCII SV	pRCII-1B	*Kpn* I 1.5 kbp	M. Bishop‡
yes†	Y73 SV	λY73-11A	*Sst* I 4.0 kbp	18
rel†	ARVT	prel	*Eco*RI 0.8 kbp	19
ski†	SKV	pvski-1	*Xho* I 2.8 kbp	Unpublished data
abl§	Abelson MuLV	pABsub3	*Hin*dIII/*Sst* II 2.0 kbp	20
fes§	Feline SV	pGA-FeSV	*Pst* I 0.5, 0.55 kbp	21
mos§	Moloney MuSV	pmos-1	*Pst* I 0.45 kbp	D. Dina‡
H-*ras*§	Harvey MuSV	pBS-9	*Eco*RI 0.5 kbp	22
		pHB-11	*Eco*RI/*Bam*HI 2.2 kbp	22
K-*ras*§	Kirsten MuSV	pHiHi-3	*Eco*RI 1.0 kbp	22
		pKBE-2	*Eco*RI/*Bam*HI 3.1 kbp	22
fms§	McDonough feline SV	λSM-FeSV	*Kpn* I 2.8, 4.8 kbp	23
sis§	Simian SV	pvsis	*Eco*RI/*Sal* I 2.1 kbp	24

The table lists the v-*onc* sequences that were tested for homology to three human transforming genes.
* SV, simian virus; ARVT, avian reticuloendotheliosis virus T; SKV, Sloan–Kettering virus; Mu, murine.
† Avian.
‡ Personal communication.
§ Mammalian.

RESULTS

Three Human Transforming Genes Have Homology to Viral *ras* Genes. Molecular clones of v-*onc* were cleaved with restriction endonucleases to separate v-*onc* and vector sequences, and triplicate aliquots of these digests were subjected to agarose gel electrophoresis and Southern nitrocellulose filter blotting. The three replica filters were hybridized at low stringency to ^{32}P-labeled recombinant DNAs containing all or part of the three different human transforming genes (Fig. 1). The transforming human genes were those isolated from the bladder carcinoma cell line T24 (Fig. 1*B*), the lung carcinoma cell line Calu-1 (Fig. 1*C*), and the neuroblastoma cell line SK-N-SH (Fig. 1*D*).

All three human transforming genes showed homology to v-H-*ras* and v-K-*ras* (Fig. 1, lanes 8–11). The human transforming genes were not homologous to nine other v-*onc* genes (Fig. 1, lanes 1–7, 12, and 13). The hybridization detected in other lanes of this figure represent hybridization between pBR322 plasmid and λ phage DNA sequences in the probes and on the filters. It is not surprising that each human transforming gene that hybridized with one also hybridized with both v-H-*ras* and v-K-*ras* because these v-*onc* genes share sequence homology and encode immunologically and structurally related proteins (22).

To explore further the homology between these genes, we hybridized each v-*ras* gene separately under conditions of high stringency to Southern blotted DNAs of the T24, SK-N-SH, and Calu-1 transforming genes and to pBR322 clones containing v-H-*ras* and v-K-*ras* (Fig. 2). As expected, v-H-*ras* hybridized well to the T24 transforming gene (Fig. 2*A*, lane c) and to the normal allele of this gene (Fig. 2*A*, lane d). The v-H-*ras* probe hybridized only weakly to a 3.0-kbp *Eco*RI restriction endonuclease fragment of the Calu-1 transforming gene (Fig. 2*A*, lane e) and to two *Eco*RI DNA fragments of the SK-N-SH transforming gene (Fig. 2*A*, lane g). In contrast, the v-K-*ras* probe was most closely related to the Calu-1 transforming gene, hybridizing to 3.1-, 3.0-, and 2.4-kbp *Eco*RI DNA fragments of this gene (Fig. 2*B*, lanes l and m). Longer autoradiography of the filter showed weak hybridization between v-K-*ras* and the T24 transforming gene (Fig. 2*C*, lanes j and k) and the two *Eco*RI fragments that comprise the SK-N-SH transforming gene (Fig. 2*C*, lane n).

In summary, all three human transforming genes shared homology to the v-*ras* genes. The T24 transforming gene was closest to v-H-*ras*, the Calu-1 transforming gene was closest to v-K-*ras*, and the SK-N-SH transforming gene was more distantly related to the v-*ras* genes.

The Lung and Colon Carcinoma Transforming Gene Is a Human Homolog of v-K-*ras*. DNAs from normal and transformed NIH 3T3 cells and from human cells were cleaved with restriction endonuclease *Eco*RI and subjected to gel electrophoresis and filter-blot hybridization, with the pKBE-2 clone

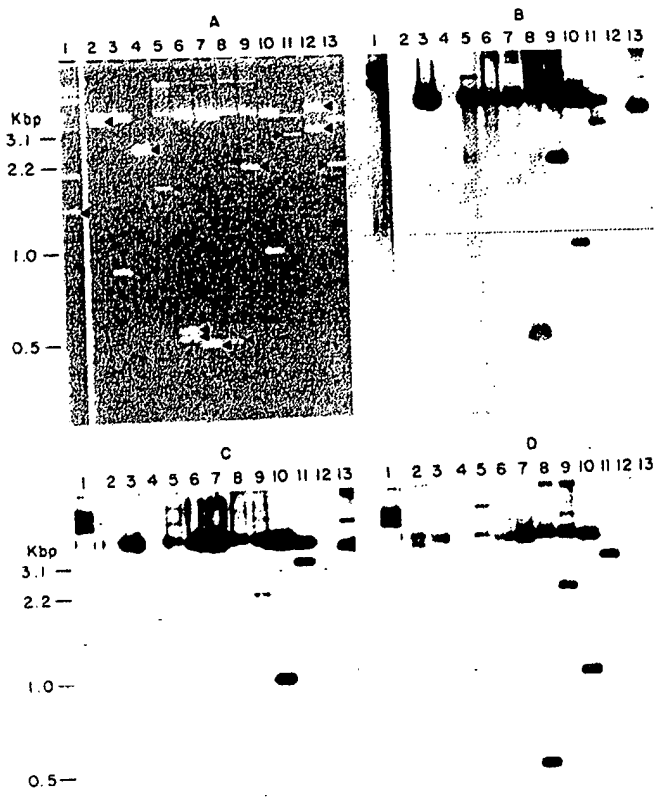

FIG. 1. Southern filter hybridization of three human transforming genes to v-*onc* DNA sequences. Molecular clones of retroviral oncogenes were digested with restriction endonucleases to separate oncogenic sequences from plasmid or bacteriophage DNA vectors. Digests were subjected to electrophoresis through 1% agarose gels, and the DNAs were transferred from gels to nitrocellulose filter papers (14). Filters were hybridized with cloned human transforming gene DNAs, which had been labeled with ^{32}P by nick translation, and filters were subsequently washed under nonstringent conditions. (*A*) Ethidium bromide stain of a gel prior to filter transfer (arrows denote restriction endonuclease fragments containing v-*onc* sequences). (*B–D*) Filter hybridizations with ^{32}P-labeled pT24 DNA (*B*), ^{32}P-labeled pLC3 (*C*), and ^{32}P-labeled λNPS-1-1-1 (*D*). Lanes show the v-*onc* DNA restriction digests: 1, v-*fps Kpn* I digest of pRCII-1B; 2, v-*yes Sst* I purified insert from λY73-11A; 3, v-*rel Eco*RI digest of prel; 4, v-*ski Xho* I purified insert from pvski-1; 5, v-*abl Hin*dIII/*Sst* I digest of pABsub3; 6, v-*fes Pst* I digest of pGA-FeSV; 7, v-*mos Pst* I digest of pmos-1; 8, v-H-*ras Eco*RI digest of pBS-9; 9, v-H-*ras Eco*RI/*Bam*HI digest of pHB-11; 10, v-K-*ras Eco*RI digest of pHiHi-3; 11, v-K-*ras Bam*HI/*Eco*RI digest of pKBE-2; 12, v-*fms Kpn* I purified inserts from λ SM-FeSV; 13, v-*sis Eco*RI/*Sal* I digest of pvsis.

of v-K-*ras* (22) used as the ^{32}P-labeled hybridization probe (Fig. 3). NIH 3T3 cells transformed with DNA from the lung and colon carcinoma cells (Fig. 3, lanes 1, 2, and 3) contain K-*ras*-related sequences not endogenous to NIH 3T3 (Fig. 3, lane 4). The newly acquired K-*ras*-related *Eco*RI fragments in these transformed cells comigrated with v-K-*ras*-related *Eco*RI fragments prominent in human DNA (Fig. 3, lane 5). These *Eco*RI fragments are 2.4, 3.0, 3.1, and ≈6.7 kbp in size. Only one high molecular weight K-*ras*-related *Eco*RI fragment in human DNA was not transferred to NIH 3T3 cells. Similar results were observed by Der *et al.* (5) in NIH 3T3 cells transformed with DNA from Lx-1, indicating that the same human K-*ras* homolog is the transforming gene of these cells.

v-K-*ras* Homologous Regions of the Calu-1 Transforming Gene. A large portion (26 kbp) of the transforming gene of the

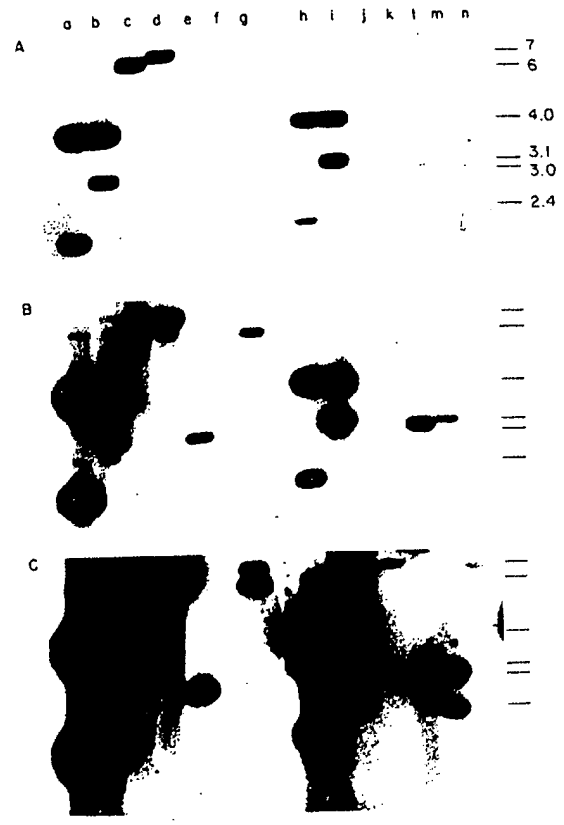

FIG. 2. Hybridization of v-*ras* sequence probes to filter-blotted human transforming gene DNAs. Cloned human transforming gene and v-*ras* gene DNAs were cleaved with restriction endonucleases, and duplicate aliquots were subjected to 1% agarose gel electrophoresis and Southern filter blotting. The filters were hybridized with either ^{32}P-labeled pHB-11 v-H-*ras* (lanes a–g) or ^{32}P-labeled pKBE-2 v-K-*ras* (lanes h–n). The filters were washed under stringent conditions. Autoradiographic exposures were for 2 hr (*A*), 12 hr (*B*), and 72 hr (*C*). Lanes: a and h, *Eco*RI/*Bam*HI pHB-11 (0.1 μg); b and i, *Eco*RI/*Bam*HI pKBE-2 (0.1 μg); c and j, *Bam*HI λT22 (1.0 μg); d and k, *Bam*HI λP3 (1.0 μg); e and l, *Eco*RI λL2-L11 (1.0 μg); f and m, *Eco*RI λL2-R7 (1.0 μg); g and n, *Eco*RI λNPS-1-1-1 (1.0 μg). Size markers are in kbp.

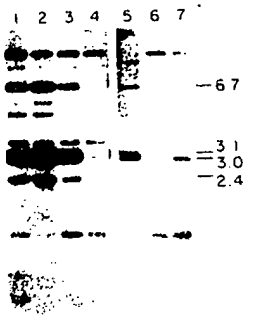

FIG. 3. Identification of lung and colon carcinoma transforming genes as K-*ras* homologs. Six micrograms of *Eco*RI-digested cellular DNA was electrophoresed through 1% agarose gels and subsequently blotted to nitrocellulose. The filters were hybridized and washed under stringent conditions with ^{32}P-labeled pKBE-2 (v-K-*ras*) as probe. Lanes: 1, NIH 3T3 transformed with SK-CO-1 DNA; 2, NIH 3T3 transformed with SK-LU-1 DNA; 3, NIH 3T3 transformed with Calu-1 DNA; 4, NIH 3T3; 5, T24; 6, NIH 3T3 with 50 pg of *Eco*RI-cleaved λL2-11 DNA; 7, NIH 3T3 with 50 pg of *Eco*RI-cleaved λL2-R7 DNA. For structure of λ clones, see Fig. 4*A*. Size markers are in kbp.

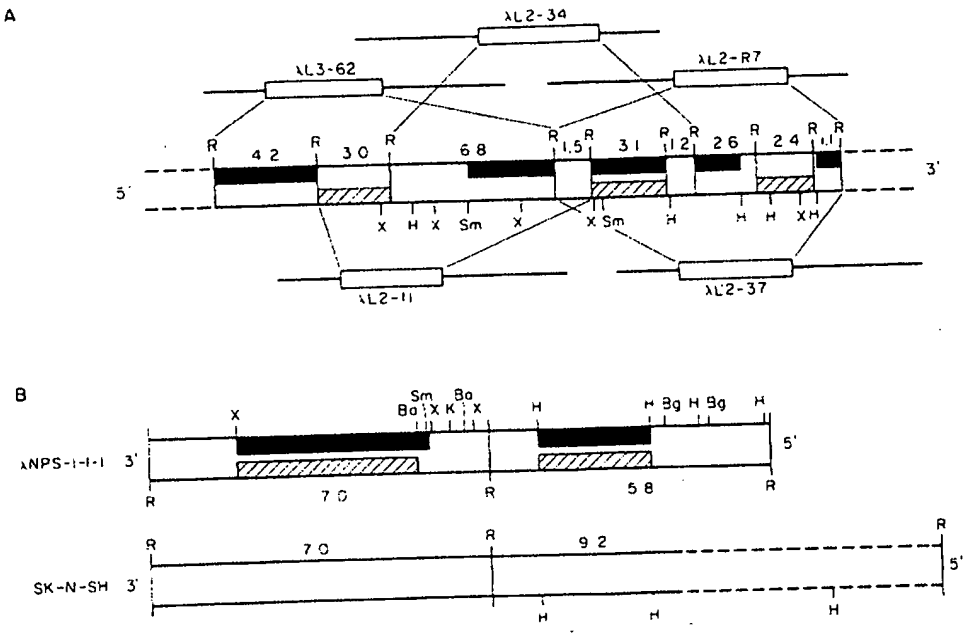

FIG. 4. Maps of *ras*-homologous regions in Calu-1 and SK-N-SH transforming genes. (*A*) Composite restriction endonuclease map of part of the Calu-1 transforming gene. The map was derived from overlapping restriction endonuclease maps of inserts in λ Charon 4A, some of which are shown above and below the map. We estimate that 30% of the gene remains to be cloned. ■, Restriction fragments that hybridize with the BLUR8 clone of human *Alu* repeat sequence DNA; ▨, restriction fragments that hybridize at high stringency to the v-K-*ras* clone pKBE-2. The 5′ and 3′ ends of the transcription unit were deduced by hybridization of the cloned Calu-1 transforming gene with 5′ and 3′ specific restriction endonuclease fragments of pKBE-2. (*B Upper*) Restriction endonuclease map of the SK-N-SH transforming gene contained within λNPS-1-1-1 is taken from Shimizu *et al.* (26). (*B Lower*) 5′ flanking sequences of *B Upper* have undergone rearrangement, showing the restriction map of the genomic SK-N-SH transforming sequence (26). ■, Restriction fragments that hybridize with the BLUR8 clone; ▨, restriction fragments that hybridize at high stringency with the v-H-*ras* clone pHB-11. The 5′ and 3′ orientations of the transcription unit were determined as above. Numbers show distances in kbp. Sites for restriction endonucleases are: Ba, *Bam*HI; Bg, *Bgl* II; H, *Hin*dIII; K, *Kpn* I; R, *Eco*RI; Sm, *Sma* I; and X, *Xba* I.

human lung carcinoma cell line Calu-1 was cloned into partially overlapping λ Charon 4A phages as described. A composite restriction endonuclease map for this gene is shown in Fig. 4*A*. Three separate regions of homology to v-K-*ras* were determined by hybridization analysis, comprising 3.0-, 3.1-, and 2.4-kbp *Eco*RI fragments (see Fig. 2, lanes l and m). All three v-K-*ras*-related *Eco*RI fragments and a fourth 6.7-kbp *Eco*RI fragment, which has not been cloned yet, were present in all NIH 3T3 cells transformed with DNA from various lung and colon carcinoma cell lines (Fig. 3, lanes 1–3, 6, and 7). These Kirsten homologous regions do not arise by tandem gene duplications because they hybridized to discrete regions of the cloned v-K-*ras* gene (data not shown). Indeed, hybridization with specific v-K-*ras* DNA fragments allowed us to make an unambiguous assignment of the direction of transcription (see Fig. 4*A*). In contrast to the small T24 transforming gene, which is entirely contained on a 2.9-kbp *Sac* I fragment (25), the transforming gene of Calu-1 is probably greater than 30 kbp.

The SK-N-SH Neuroblastoma Transforming Gene Is a New Member of the *ras* Gene Family. Although the SK-N-SH neuroblastoma transforming gene was weakly homologous to both v-H-*ras* and v-K-*ras*, we reasoned that it may encode a protein structurally and serologically related to Harvey and Kirsten *ras* gene product. We tested this possibility by using a broadly reactive monoclonal antibody against *ras*-encoded protein to immunoprecipitate [^{35}S]methionine-labeled extracts from three independently derived NIH 3T3 transformants containing the SK-N-SH transforming gene. Immune precipitates from these cells and from NIH 3T3 transformed by Harvey sarcoma virus unintegrated DNA, NIH 3T3 transformed by DNAs from human lung and colon carcinoma cells, and NIH 3T3 itself were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 5). A protein with an apparent M_r of 19,000 was seen in immunoprecipitates of v-H-*ras*-transformed NIH 3T3 (Fig. 5, lane 2) but not in NIH 3T3 controls (Fig. 5, lane 1). A similarly migrating protein was seen in NIH 3T3 cells transformed with either

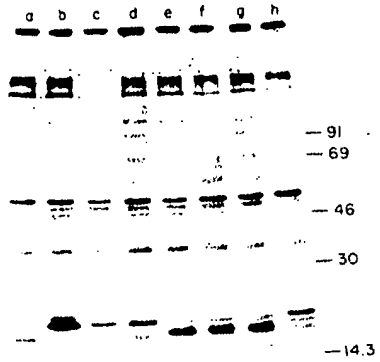

FIG. 5. *ras*-Related proteins in NIH 3T3 transformed cells. Cells (5×10^6) were labeled for 18 hr with 80 μCi of [^{35}S]methionine and extracted with nonionic detergents, and cleared lysates were used for immunoprecipitation with rat anti-*ras* p21 monoclonal antibody Y13-259 (16). Immunocomplexes were collected onto *S. aureus* protein A, dissolved and boiled in NaDodSO$_4$ sample buffer, and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel. Proteins were visualized by fluorography. Lanes: a, NIH 3T3; b, HT14B; c and d, two NIH 3T3 lines independently transformed with Calu-1 DNA; e–g, three NIH 3T3 lines independently transformed with SK-N-SH DNA; h, one NIH 3T3 line transformed with SK-CO-1 DNA. A ^{14}C-labeled protein mixture (Amersham) provided M_r standards (shown $\times 10^{-3}$).

Calu-1 or SK-CO-1 DNA (Fig. 5, lanes 3, 4, and 8). A uniquely migrating protein with an apparent M_r of 17,500 was seen in each NIH 3T3 transformant containing the SK-N-SH transforming gene (Fig. 5, lanes 5–7). This protein had an isoelectric point similar to that found for the v-H-*ras*-encoded protein (data not shown). Our findings indicate that the SK-N-SH neuroblastoma transforming gene is another member of the *ras* gene family.

We exploited the homology between the SK-N-SH transforming gene and the v-*ras* genes to determine its direction of transcription and approximate regions of homology (see Fig. 4*B*).

DISCUSSION

Three different human transforming genes that can be detected by the NIH 3T3 transformation assay are members of the *ras* gene family. The transforming gene of a bladder carcinoma cell line (T24) is a human homolog of the v-H-*ras* gene (5, 10, 11). The transforming gene of Lx-1, a human lung carcinoma cell line, is a human homolog of v-K-*ras* (5). Comparison of the work of Der *et al.* (5) with ours indicates the presence of the same transforming gene in two lung carcinoma cell lines (SK-LU-1 and Calu-1) and in one colon carcinoma cell line (SK-CO-1). This same gene is also detectable by DNA transfer in human lung and colon tumors maintained in *nude* mice (unpublished data) and in the colon carcinoma cell line SW480 (3, 9). The transforming gene of a human neuroblastoma cell line (SK-N-SH) is related to (but distinct from) the homologs of the v-H-*ras* and v-K-*ras* genes and represents a third branch within the *ras* gene family. Each branch may have more recent evolutionary offshoots. Thus, Chang *et al.* (34) reported two human homologs of v-H-*ras* (H-*ras*-1 and -2) and two homologs of v-K-*ras* (K-*ras*-1 and -2). A comparison of restriction endonuclease maps for these genes with the three human transforming genes we have isolated indicates that the T24 bladder carcinoma-transforming gene is H-*ras*-1, the lung and colon carcinoma-transforming gene is probably K-*ras*-2, and the SK-N-SH neuroblastoma-transforming gene is a heretofore uncharacterized gene. We propose calling the human transforming gene of SK-N-SH the N-*ras*-1 gene.

It is of considerable interest that a wide variety of tumor cells contain activated *ras* genes, detectable by gene transfer into NIH 3T3 cells. Several factors possibly contribute: *ras* transforming genes may be more readily detected than other transforming genes by the NIH 3T3 focus assay; *ras* genes may be easily activated by mutation; and *ras* genes may have critical cellular functions in a wide variety of cell types. The function of the *ras* gene products is not known nor is it known whether they perform physiologically distinguishable roles. However, it is known that an altered amino acid sequence is responsible for the activation of the H-*ras*-1 gene of T24 (25, 35, 36), and we speculate that alteration in the *ras* gene products may be a common step in many forms of human cancer.

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